Natural Killer Cell:

Repertoire Development, Education and Activation

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ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BLT	Bone marrow–liver–thymus
BRG	BALB/c RAG2$^{-/-}$ $\gamma_c^{-/-}$
cDC	Conventional dendritic cell
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
γc	Common gamma chain
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DZ	Dendritische Zellen
EAT2	EWS/FLI1-activated transcript 2
EBV	Epstein Barr virus
EVT	Extravillous trophoblast
f-actin	Filamentous actin
FcεRIγ	High-affinity IgE receptor subunit-γ
GM-CSF	Granulocyte macrophage colony-stimulating factor
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
HLA	Human leukocyte antigen
HCV	Hepatitis C virus
HFL	Human fetal liver
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
huMice	Humanized mice
huNSG	Humanized mice on NSG background
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IM	Infectious mononucleosis
IS	Immunological synapse

ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer cell immunoglobulin-like receptor
KLRG1	Killer cell lectin-like receptor G1
LIR	Leukocyte immunoglobulin-like receptor
LRC	Leukocyte receptor complex
MHC	Major histocompatibility complex
MTOC	Microtubule-organizing center
NCR	Natural cytotoxicity receptor
NK	Natural Killer, Natürliche Killer
NOD	Non-obese diabetic
NSG	NOD-scid $\gamma_c^{-/-}$
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PILR	Paired Ig-like 2 receptor
PLZF	Pro-myelocytic leukemia zinc finger
PRR	Pattern recognition receptor
PTLD	Post-transplant lymphoproliferative disease
Rag	Recombination-activating gene
RIU	Raji-infecting unit
SLO	Secondary lymphoid organ
SMAC	Supramolecular activation cluster
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VLP	Virus like particle
YFV	Yellow fever vaccine

CHAPTER ONE

GENERAL SUMMARY

1.1 Zusammenfassung

Natürliche Killer (NK) Zellen spielen eine wesentliche Rolle bei der angeborenen Immunantwort des Menschen. Dank ihres frühzeitigen Eingreifens bei viralen Infekten sowie ihrer Fähigkeit transformierte Zellen zu erkennen, sind sie eine effiziente erste Instanz in der Immunabwehr. Zudem unterstreichen aktuelle Studien immer häufiger nicht nur ihren Stellenwert während der prompten Immunantwort, sondern auch als Bestandteil des langlebigen immunologischen Gedächtnisses. Dementsprechend wird es immer wichtiger, die Einzelheiten zu verstehen, welche die NK Zell Biologie steuern.

In unserer ersten Studie haben wir ein neues humanisiertes Maus Modell entwickelt, anhand dessen wir den Einfluss von HLA Klasse I Molekülen in der Entwicklung von NK Zellen beobachten konnten. Für eine Gruppe von NK Zell Rezeptoren, den sogenannten killer cell immunoglobulin-like receptors (KIRs), fungieren bestimmte HLA Klasse I Moleküle als Liganden (KIR-Liganden). Diese Interaktionen sind für einen als NK Zell Erziehung genannten Reifungsprozess unerlässlich. Für unsere Studie haben wir zwei Spender, welche ungleich waren bezüglich ihrer KIR-Liganden Ausstattung, in einzelne Empfängermäuse transplantiert. Immunzellen beider Spender wuchsen in ähnlichen Frequenzen in den Tieren an und konnten über den gewählten Beobachtungszeitraum miteinander koexistieren. Das KIR Repertoire der rekonstituierten NK Zellen veränderte sich weder im Normalzustand noch während einer experimentellen Epstein Barr Virus (EBV)-Infektion im Vergleich zum KIR Repertoire der NK Zellen des Spenders, sondern blieb sehr divers, wie es in Neugeborenen zu finden ist. Des Weiteren konnten wir eindeutig belegen, dass in unserem Modell die Anwesenheit von fremden HLA Liganden den Prozess der NK Zell Erziehung aufhebt. Dies würde gegen das sogenannte Bewaffnungsmodel sprechen und für eine als Entwaffnung bezeichnete Hypothese. Weiterhin konnten wir beobachten, dass NK Zellen, welche nicht durch den Erziehungsprozess gegangen sind, trotzdem noch körpereigene Zellen erkennen konnten und diese entsprechend nicht angreifen. Ferner konnten wir feststellen, dass EBV infizierte Tiere, welche mit zwei Spendern transplantiert waren, niedrigere virale Lasten hatten. Dies könnte darauf hinweisen, dass die nicht-erzogenen NK Zellen wesentlich an der Immunantwort gegen EBV beteiligt sind, ähnlich wie es bereits für die Infektion mit murinem Zytomegalovirus gezeigt wurde. Nachdem wir die NK Zellen in den Tieren depletiert haben, hob sich der beobachtete Effekt der doppelten Transplantation, vor EBV zu schützen, wieder auf, was unsere Theorie bekräftigt. Diese Studie hat demzufolge neue

Erkenntnisse dazu erbracht, wie sich NK Zellen in einem veränderten HLA Umfeld entwickeln und welche funktionellen Konsequenzen dies ergibt.

Im Mittelpunkt des zweiten Projekt stand die Beihilfe der plasmazytoiden dendritischen Zelle (DZ) für NK Zellen. Vorangehende Daten haben gezeigt, dass konventionelle DZs eine sogenannte regulatorische Synapse mit NK Zellen eingehen, weshalb wir in dieser Studie nun auch die Rolle und Strukturen der Synapse zwischen plasmazytoiden DZs und NK Zellen besser verstehen lernen wollten. Wir fanden, dass Rezeptoren und Aktin zur Synapse hin polarisieren und die Interaktion zwischen den Zellen stabilisieren. Zudem konnten wir zeigen, dass phosphorylierte STAT Proteine ebenfalls an der Synapse angereichert waren, was darauf hinweist, dass es sich um eine aktivierende Synapse handelt. Ferner konnten wir bestätigen, dass eine Interaktion zwischen den beiden Zellpopulationen zur Aktivierung und Hochregulation von Zytotoxizität in den NK Zellen führte. Obwohl die Synapse alle Kennzeichen einer zytotoxischen Synapse vorwies, wurden die plasmazytoiden DZs interessanterweise nicht angegriffen. NK Zellen scheinen also imstande zu sein eine einzigartige immunologische Synapse mit plasmazytoiden DZs einzugehen.

1.2 Summary

Natural killer (NK) cells are an instrumental part of the human innate immune response. Their intervention at the primary stages of viral infections as well as detection of altered-self makes them an efficient first line of defense. Moreover, current studies have started to highlight their contribution not only as early responders but also as long-lived memory cells. Therefore, understanding the details of NK cell biology is of growing importance. One of the poorly understood developmental aspects in human NK cell biology is the impact and manner of the interaction of killer cell immunoglobulin-like receptors (KIRs) with their ligands, HLA class I molecules, in shaping the KIR repertoire itself and also during the process of NK cell education.

In a first study that aimed to understand the influence of HLA haplotypes on NK cell development and repertoire diversity we fashioned a novel humanized mouse model by co-reconstituting two KIR-ligand mismatched donors into a single mouse recipient. We found that both compartments were adept at engrafting the animals at equal frequencies and persisted side-by-side. Furthermore, as compared to the KIR repertoire found in the donor(s), neither in steady state nor under Epstein Barr virus (EBV)-induced inflammatory conditions were there any alterations to the KIR repertoire which remained extremely diverse, as found in human neonates. Moreover, we were able to show that the presence of non-cognate HLA completely abolishes education of NK cells, thus arguing in favor of the disarming hypothesis. Additionally, we demonstrated that loss of education does not impair the NK cell's ability to discriminate self, i.e. non-educated NK cells downregulate cytotoxicity when KIRs get engaged by self HLA. Interestingly, we observed lower viral burdens in co-reconstituted animals, which could point towards a possible involvement of non-educated human NK cells in controlling EBV infection, as has been shown for murine cytomegalovirus (MCMV). This theory was further supported by data from co-reconstituted animals that had been depleted of NK cells and concomitantly lost their protection from EBV. Thus, this study has provided novel insights into the development and function of NK cells under the influence of mismatched HLA.

The second project focused on the role of plasmacytoid dendritic cells (DCs) as accessory cells in NK cell-mediated immune response. As NK cells had been shown to form distinct regulatory synapses with conventional DCs, we investigated the function and structure of the synapse between plasmacytoid DCs and NK cells. Our results showed

that polarization of receptors as well as actin takes place, thus stabilizing the interaction between the cells. In addition we were able to demonstrate accumulation of phosphorylated STAT proteins at the synapse, which points toward an activating nature of the synapse. We confirmed that the interaction leads to activation as well as upregulation of cytotoxicity in NK cells. Intriguingly, although the synapse showed all the hallmarks of a cytotoxic synapse, plasmacytoid DCs were not killed. Hence, NK cells seem capable of forming a unique type of immunological synapse with plasmacytoid DCs.

CHAPTER TWO

INTRODUCTION

PART I– HUMAN IMMUNE SYSTEM

The human immune system is made up of various cell populations that in a complex interplay jointly protect us from pathogens and transformed cells. The two branches of the immune system are separated into innate and adaptive arms. The innate immune system encompasses defense mechanisms that are broadly specific and encoded in the host's germ line. These are for instance physical barriers such as the mucosal tissue lining our airways and gut, or secreted bioactive small molecules. In addition, the first response is aided by a number of innate leukocytes, which include macrophages, dendritic cells, granulocytes and innate lymphoid cells. These cells possess receptors on their surface, which are able to recognize specific molecular patterns in their environment. In contrast, T- and B-lymphocytes, which comprise the adaptive immune system, express receptors highly specific toward the antigen they recognize. This collection of receptors is generated by somatic gene rearrangement and hypermutation, permitting the formation of millions of different antigen receptors, each with a unique specificity. Furthermore, it has been well established that the lymphocytes of the adaptive immune system are able to form a durable pool of memory cells, which allow for improved response time and magnitude when antigens are encountered repeatedly. Although the innate and adaptive immune systems are often described as two separate arms of the host response, in reality they work together to achieve a comprehensive immune response with the innate immune system in charge of the initial defense as well as guiding the adaptive response which catches on later after the antigen-specific cells have undergone clonal expansion. Thus, a synergy between them is essential for the host to mount a fully effective immune response (Chaplin, 2010).

PART II– NATURAL KILLER CELLS

Natural Killer (NK) cells are the prototypic innate lymphoid cell, and were first discovered in the mouse as lymphocytes that were able to spontaneously lyse transformed cells (Kiessling et al., 1975). Soon after the same observation was made in humans, where the T cell-depleted fraction of lymphocytes isolated from peripheral blood was incubated with human and xenogeneic cell lines. Lysis of the target cells was observed, and the process was termed spontaneous lymphocyte-mediated cytotoxicity at

the time (Jondal & Pross, 1975; Pross & Jondal, 1975). At this point, it was already apparent that the human cell line K562, originally isolated from a chronic myelogenous leukemia patient, constituted a good NK cell target (Lozzio and Lozzio, 1975). Only later though would it become clear, that the lack of MHC class I expression and upregulation of stress ligands on their cell surface, was what made them such highly sensitive *in vitro* targets (Drew et al., 1977; Saksela et al., 1979). In an effort to characterize these mysterious cytotoxicity-mediating lymphocytes, a number of isolation methods and culturing techniques were tested. With the results obtained from these trials, it was evident that these lymphocytes did not match any of the populations known at the time and they were termed N-cells (Herberman et al., 1975b). Further work demonstrated their involvement not only in targeting transformed cells, but also in responding to viral infections (Trinchieri and Santoli, 1978). This ability to rapidly counter viral infections as well as efficiently eradicate transformed cells, in short to naturally kill targets, is what finally gave them their name as natural killer cells.

Several ligand-receptor interactions can trigger NK cell activation, mostly combinations thereof (Long et al., 2013), such as lack of MHC class I interaction with inhibitory receptors, binding of NKG2D to its various ligands, DNAM-1 recognition of PVR and Nectin-2 or antibody-dependent cell-mediated cytotoxicity (ADCC). In fact, NK cells are the main mediators of ADCC, a process where infected cells are opsonized by specific antibodies that bind to the antigens expressed on their surface. The CD16 receptor on NK cells is subsequently able to recognize the Fc part of these antibodies and initiate killing. To induce target cell lysis, NK cells take advantage of cytotoxic proteins, which are stored within specialized secretory lysosomes also termed lytic granules. The abundantly present cytotoxic proteins within secretory lysosomes are the granzymes and perforin. Once the NK cell receives the signal to kill, it initiates exocytosis and release of the cytotoxic granules across the immunologic synapse, thus limiting the spread of the cytotoxic effector proteins. In this process the cytotoxic proteins work together to facilitate killing of the target. Perforin mediates the formation of pores thus enabling the entry of granzymes that cleave a variety of targets in the cytoplasm, finally resulting in cell death (Topham & Hewitt, 2009). However, in order to be able to elicit their effector functions, NK cells need to undergo the additional developmental process of education allowing them to tune their reactivity in a way that they are non-reactive to self and specifically detect transformed cells.

2.1 Receptors Shaping NK Cell Biology

For NK cells, diversity and specificity does not arise from a single polymorphic receptor but rather emerges from the large variety of receptors many of which are differentially expressed on individual NK cells (Figure 1). Since NK cells lack the degree of antigen specificity other lymphocytes possess, they rely on a series of activating and inhibitory receptors to regulate their immune responses towards targets. Unlike T and B cells, they are unable to achieve diversity of their receptors by somatic gene rearrangement. However, some level of flexibility has been achieved through the rapid genetic evolution of the NK cell recognition receptor families as well as promiscuity of ligand binding (McQueen & Parham, 2002). There are three major families of NK cell receptors - the KIRs, NKG2s and NCRs. Together with other receptors on the surface of NK cells, they all contribute to balance the immune response of NK cells.

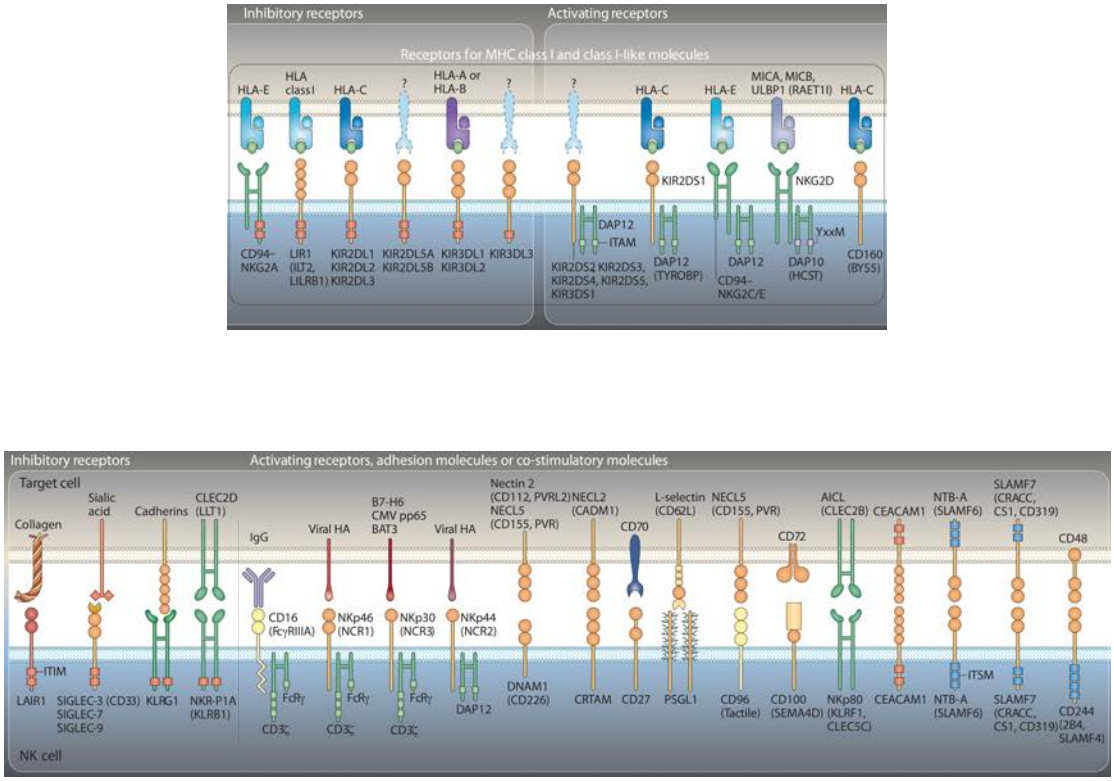

Figure 1. Adapted from key receptors on human NK cells ("NK cells: receptors and functions," 2010)

2.1.1 Killer Immunoglobulin-like Receptors

Killer immunoglobulin-like receptors (KIRs) evolved from the immunoglobulin-superfamily (Ig-superfamily) and are built-up of type 1 transmembrane glycoproteins with two or three Ig-like domains (Colonna & Samaridis, 1995; Wagtmann et al., 1995). Their nomenclature is based on the number of extracellular Ig-domains (KIR2D or KIR3D) and on an either long (L) or short (S) cytoplasmic tail (E. O. Long, Colonna, & Lanier, 1996). The KIR family comprises both activating (short cytoplasmic tail) and inhibitory (long) receptors that signal through association with DAP-12 via its immunoreceptor tyrosine-based activation motif (ITAM) or through their own immunoreceptor tyrosine-based inhibition motif (ITIM), respectively (Burshtyn et al., 1996; Campbell, Dessing, López-Botet, Cella, & Colonna, 1996; Lanier, 1998). As the KIR repertoire for each individual is unique, the interplay of activating and inhibitory KIRs can be of importance in a person's immune response. Thus, KIR activating genes are commonly associated with increased susceptibility to autoimmunity and protection against infection while inhibitory genes have been mainly implicated in the opposite, i.e. susceptibility to infection and protection against autoimmunity (Augusto, Lobo-Alves, Melo, Pereira, & Petzl-Erler, 2012). Moreover, the KIR haplotype of an individual can contribute beneficially to NK cell immunity or reproductive fitness as discussed below (Khakoo et al., 2004; Parham & Moffett, 2013).

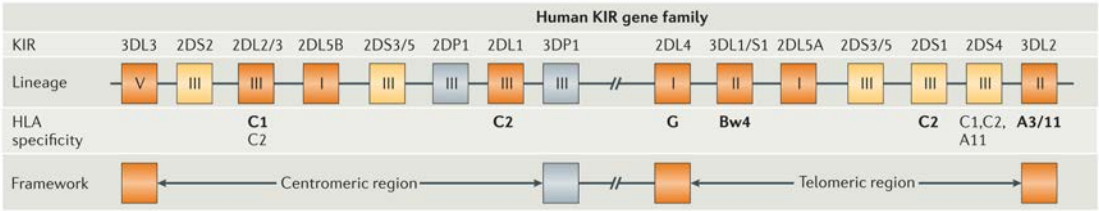

Figure 2. Humans KIRs recognize four epitopes of HLA-A, HLA-B and HLA-C (Parham & Moffett, 2013)

KIRs, like their classic MHC class I ligands which are also called human leukocyte antigens (HLAs), are encoded by a genetic complex located on chromosome 19, termed leukocyte receptor complex (LRC) (Kelley, Walter, & Trowsdale, 2005; M. J. Wilson et al., 2000). There are 13 expressed KIR genes and two KIR pseudogenes encoding for dedicated activating receptors KIR2DS1, 2, 3, 4, 5 and inhibitory receptors KIR2DL1,

2DL2/3 and 2DL5 (Figure 2). In addition, exceptional receptors, such as KIR2DL4 have the potential to be both activating and inhibitory, while KIR3DL1/S1 has two mutually exclusive subsets of allotypes that allows it to have either activating or inhibitory functions (Campbell & Purdy, 2011).

The repertoire of KIR genes expressed within one individual defines a KIR haplotype which differs on the basis of KIR gene content at the KIR locus (Pyo et al., 2010; Uhrberg et al., 1997). Three conserved genes (KIR3DL3, 2DL4 and 3DL2) are common to all haplotypes and pinpoint two regions of gene-content diversity in the centromeric and telomeric part of the locus (A. M. Martin et al., 2004). KIR haplotypes are divided into two groups; the A haplotype which is enriched for inhibitory KIRs that bind HLA class I and only expresses one activating gene (KIR2DS4) and the B haplotype that is characterized by activating and inhibitory KIRs of which some have lost or lack the capacity to bind HLA (Uhrberg, Parham, & Wernet, 2002; M. J. Wilson et al., 2000). Some receptors that are common to both haplotypes show functional differences between allotypes of the A and B KIR haplotypes (Bari et al., 2009). One particularly good example is the KIR3DL1/DS1 gene, which in its inhibitory form in A haplotype binds the Bw4 epitope, whereas B haplotype KIR3DS1 does not (Parham, Norman, Abi-Rached, & Guethlein, 2011).

Diversity of KIR genes is achieved by unusual presence/absence polymorphism and reciprocal combination between the central region of the KIR locus 3DP1 and 2DL4 (Figure 2) (Uhrberg et al., 1997). This recombination allows re-shuffling of the centromeric and telomeric gene-content motifs resulting in haplotypes that combine an A haplotype with a B haplotype. Conventionally any such recombinant haplotypes have been termed B haplotypes, reserving the term A haplotype for haplotypes entirely consisting of A motifs (Hsu et al., 2002; Pyo et al., 2010). Moreover, allelic variants of KIR genes influence the expression levels and patterns of KIRs, giving rise to different NK cell populations that express multiple different assortments of KIRs (Gardiner, 2008). Surprisingly, the mechanism by which NK cells decide what KIRs are expressed on their surface is still elusive. However, it has been observed that the overall KIR repertoire is determined by the KIR genotype and expression is regulated by methylation of KIR gene loci (Chan et al., 2003; Yawata et al., 2006). Furthermore, some studies have found evidence that expression of HLA class I is able to subtly modulate KIR expression as well (Shilling et al., 2002; Sleiman et al., 2014). In addition, evidence suggests that the KIR repertoire can be significantly skewed by exposure to pathogens, as in HCMV-seropositive donors compared to HCMV-negative individuals (Béziat et al., 2013;

Schönberg et al., 2011). Thus several mechanisms ensure the formation of a diverse NK cell pool that can interact with its environment.

MHC class I binding is one way that NK cells can survey their surroundings. The genetic complex encoding MHC class I members, called HLA complex and located on chromosome 6, is the most highly polymorphic segment of the human genome (1000 Genomes Project Consortium et al., 2010; Robinson, Mistry, McWilliam, Lopez, & Marsh, 2010). Classical HLA class I ligands are expressed on the surface of nucleated cells and consist of an alpha chain with three extracellular domains (α1, α2 and α3) and beta-2 microglobulin (Figure 3). Their key function is to present peptide fragments to immune cells, which they bind in a groove formed by the α1 and α2 domains {Rammensee:1993eo}. Within the family of HLA class I genes, HLA-A, -B, -C are highly polymorphic while HLA-E, -F, -G are more conserved. Some KIRs bind to members of the first group, and HLA-E interacts with the NKG2A/C receptors (see below), while HLA-G is implicated in mediating NK cell functions during early pregnancy by binding to KIR2DL4 (Lash, Robson, & Bulmer, 2010). Only HLA-F remains enigmatic, possibly acting as a chaperone that retrieves unfolded HLA class I molecules from the plasma membrane and escorts them inside the cell (Goodridge, Burian, Lee, & Geraghty, 2010).

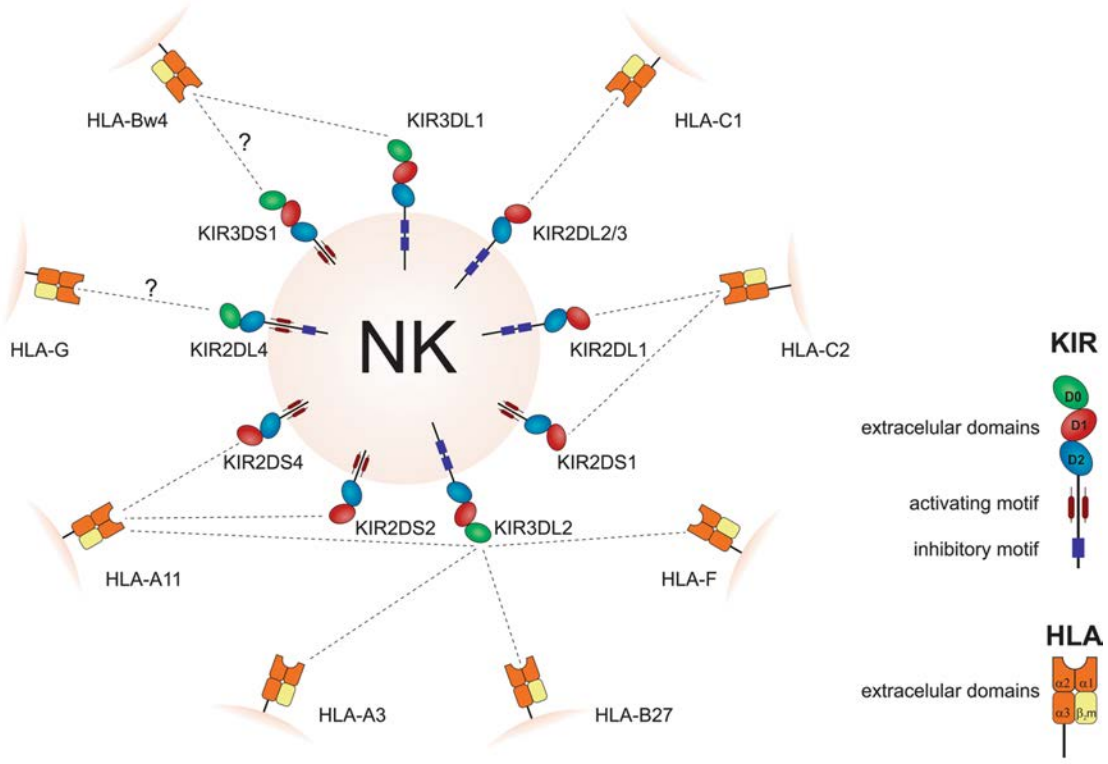

Figure 3. Interaction of KIR with their known HLA ligands (Augusto & Petzl-Erler, 2015)

Structural studies have shown that KIRs which bind to HLA do so at the peptide-binding region, and thus have an overlapping binding site with the αβ T cell receptors (TCR) (Boyington & Sun, 2002). Competition between KIR and αβ TCR could therefore influence the selection pressure on HLA class I; i.e. HLA class I variants selected for their beneficial T cell response against one infection might induce unfavorable consequences for the NK cell response towards another pathogen, and vice versa. However this issue is likely overcome since KIRs only bind a fraction of the HLA-A, -B and -C variants. Hence, while all HLA-C allotypes have either the C1 or C2 epitope and are all KIR ligands, only 30-40% of the HLA-A and HLA-B allotypes can be bound by KIRs (Gonzalez-Galarza, Christmas, Middleton, & Jones, 2011; Older Aguilar et al., 2010; Older Aguilar, Guethlein, Hermes, Walter, & Parham, 2011). Therefore, the majority of HLA-A and HLA-B allotypes are not KIR ligands and are free to evolve exclusively under pressure from the TCRs.

Although KIR-HLA interactions are one of the central receptor-ligand cross-talks shaping NK cell biology, not all KIRs bind HLA. Only some of the 13 KIRs actually recognize HLA:

KIR receptor	**HLA class I ligand**	**Reference**
KIR3DL2	A3/A11	(Döhring, Scheidegger, Samaridis, Cella, & Colonna, 1996)
KIR3DL1 (but not KIR3DS1)	Bw4	(Carr et al., 2007; Cella, Longo, Ferrara, Strominger, & Colonna, 1994)
KIR2DL1 and DS1	C2	(Biassoni et al., 1997)
KIR2DL2/3	C1 and C2 low affinity	(Pende et al., 2009)
KIR2DS4	C1, C2 and A11	(Graef et al., 2009)
KIR2DS2	A11	(J. Liu, Xiao, Ko, Shen, & Ren, 2014)

Table 1. KIR receptors and their respective HLA ligands

For the remaining KIR2DS3, 2DS5, 2DL5, 3DS1, 3DL3, 2DP1 and 3DP1, the so-called orphan receptors, no ligands have been identified yet. To determine the unexplained functional benefits of orphan KIRs, studies were done assessing KIR genes in various populations (Gonzalez-Galarza et al., 2011). All KIR genes were represented at

significant frequencies across all populations, implying that orphan KIRs must serve some purpose that might not necessarily involve interaction with HLA class I. One example of a possible alternative pathway for the orphan KIRs is KIR3DL2, which on top of binding HLA-A3/A11 can also recognize microbial products such as CpG oligodeoxynucleotides (ODN). The presence of CpG ODN leads to downregulation of KIR3DL2 from the cell surface and co-internalization of the bound product, making it accessible to Toll-like receptors (TLRs) in early endosomes (Sivori et al., 2010a; Sivori, Falco, Moretta, & Moretta, 2010b).

For some KIR-HLA interactions, peptide sensitivity additionally plays a role in KIR binding. KIR3DL2 serves as an example that was so far only found to be able to bind HLA-A3 or -A11 if a peptide fragment derived from Epstein-Barr virus (EBV) is present in the peptide-binding groove (Hansasuta et al., 2004). In comparison, HLA-C-specific KIRs are less selective and are compatible with around 40% of all peptides that bind to HLA-C (Fadda et al., 2010; Mandelboim, Wilson, Valés-Gómez, Reyburn, & Strominger, 1997; Rajagopalan & Long, 1997; Zappacosta, Borrego, Brooks, Parker, & Coligan, 1997).

In an attempt to better understand the evolution of KIRs, several studies worked on characterizing KIR genes in simian primates such as chimpanzees. As our close ancestor, many elements of the KIR locus are similar to humans (Khakoo et al., 2000). Yet some of the most striking differences are that all their variable gene content is located in the centromeric region of the KIR gene locus and that chimpanzees only have one KIR haplotype (Abi-Rached, Moesta, Rajalingam, Guethlein, & Parham, 2010). Furthermore, most NK cell receptors of chimpanzees are HLA-C receptors that preferentially bind HLA-C2 (Moesta, Abi-Rached, Norman, & Parham, 2009; Moesta et al., 2010). This makes their system more robust compared to humans, where only half of the receptors even bind HLA class I. In addition, humans developed two haplotypes of which the A haplotype resembles the one found in their ancestor while the B haplotype has accumulated genes that encode KIRs with a reduced or lacking ability to bind HLA class I (Parham, Norman, Abi-Rached, & Guethlein, 2012).

The discrepancy found in the human KIR haplotypes in mediating immunity may well be ascribed to the role that NK cells and KIR receptors play during pregnancy. Complications during pregnancy such as preeclampsia, spontaneous abortion and fetal growth restriction are linked to cases where the mother is homozygous for group A KIR haplotypes and the fetus expresses HLA-C2 (Hiby et al., 2008; 2004). Interactions between the fetal C2 on extravillous trophoblast cells (EVTs) and maternal KIR2DL1 on

uterine NK cells are therefore the likely cause for spontaneous abortion. Accordingly, expression of the activating KIR2DS1 in maternal B KIR haplotypes is protective against these complications, allowing EVTs to successfully mediate placentation (Hiby et al., 2010). In line with these findings, the frequencies of C2 and group A KIR haplotypes within a population are inversely correlated to each other (Parham et al., 2012). As a result, pressure from human reproduction might have driven the evolution of the B KIR haplotype to attenuate such interactions during fetal development. However, the KIR A haplotype is still vitally important as evidence points to it being involved in the defense against infections. This is exemplified by KIR3DL2/3 interaction with HLA-C1 inferring protection during HCV infection or in HIV where certain allelic combinations of KIR3DL1 and HLA-B can influence the disease progression and outcome (Khakoo et al., 2004; M. P. Martin et al., 2007). Thus in summary, the human KIR system has evolved to accommodate immunity against pathologies as well as reproductive fitness.

2.1.2 CD94–NKG2 heterodimer receptors and NKG2D

The CD94-NKG2A/B/C/E heterodimers are part of the C-type lectin family of receptors. They are specialized to recognize the non-classical MHC class I molecule HLA-E on the surface of potential targets (Borrego, Ulbrecht, Weiss, Coligan, & Brooks, 1998; Braud et al., 1998; Lieto, Maasho, West, Borrego, & Coligan, 2006) and their expression levels are modulated by the secretion of cytokines in their surrounding (Bertone et al., 1999; Derre et al., 2002; Mingari et al., 1998). The ubiquitously found HLA-E is capable of binding peptides derived from the leader sequence of classical MHC class I molecules and thus presents a reflection of classical MHC class I molecules expressed on the cell (Borrego et al., 1998; N. Lee, Goodlett, Ishitani, Marquardt, & Geraghty, 1998). Highly specific binding of HLA-E through the CD94-NKG2 heterodimer therefore allows NK cells to indirectly monitor the expression of MHC class I on the target cell (Braud, Jones, & McMichael, 1997). Similar to the KIRs, the family of CD94-NKG2 receptors also has activating and inhibitory receptors. CD94-NKG2C and CD94-NKG2E have been shown to associate with DAP-12 and are thought to be activating receptors (Borrego et al., 1998; Lanier, Corliss, Wu, & Phillips, 1998), while ITIM-containing CD94–NKG2A and CD94–NKG2B receptors are inhibitory (Carretero et al., 1997; Lieto et al., 2006; Palmieri et al., 1999). As a surrogate of function, expression of NKG2C has been implicated in memory formation (Gumá et al., 2004) while NKG2A surface expression seems to delineate a progression

from early differentiation to late differentiation (Béziat, Descours, Parizot, Debré, & Vieillard, 2010).

Although the NKG2D receptor shares the same nomenclature as the CD94-NKG2 heterodimers, its function and structure are distinct. NKG2D receptors recognize stressed cells and are therefore important for NK cell-mediated control of transformed cells or cells under viral pressure (Champsaur & Lanier, 2010). In contrast to the rest of its family, NKG2D does not form a heterodimer with CD94, but rather is expressed as a homodimer, which signals through the DAP-10 or DAP-12 molecules (S. Bauer et al., 1999b; J. Wu et al., 1999). In humans, only the long isoform of NKG2D exists, which associates with four DAP-10 chains to induce both perforin-dependent cytotoxicity and cytokine-mediated responses (Billadeau, Upshaw, Schoon, Dick, & Leibson, 2003; Garrity, Call, Feng, & Wucherpfennig, 2005; Hayakawa et al., 2002; J. Wu et al., 1999). Ligands of NKG2D include the MHC class I-like transmembrane proteins MIC-A, MIC-B and ULBP4 as well as the GPI-anchored proteins ULBP1-3 (S. Bauer et al., 1999b; Chalupny, Sutherland, Lawrence, Rein-Weston, & Cosman, 2003; Cosman et al., 2001). None of these ligands are expressed on healthy cells, but are regulated by DNA damage, heat shock response pathways and genotoxic or cellular stress which are typical hallmarks of viral and malignant transformation (González, Groh, & Spies, 2006; Groh et al., 1996; Jolly & Morimoto, 2000). Thus, clearance of stressed or malignant cells is essentially facilitated by NKG2D-mediated recognition. In fact, it has such a central part in the immune response to viruses and tumors that is has become the target of immune evasion strategies. For example HCMV-infected cells produce the protein UL16 which enables the retention of several stress-ligands intracellularly (Valés-Gómez, Browne, & Reyburn, 2003) and some tumors secret either transforming growth factor-b1, which leads to downregulation of NKG2D on NK cells (Castriconi et al., 2003) or NKG2D ligands as decoys (Groh, Wu, Yee, & Spies, 2002; Salih, Rammensee, & Steinle, 2002). The latter however is not exclusively immune evasive as a recent report highlighted the role of shed NKG2D ligands in mice, supporting immunosurveillance and tumor rejection by NK cells (Deng et al., 2015) .

2.1.3 Natural cytotoxicity receptors

NKp46, NKp30 and NKp44 are all part of an additional group of activating receptors, the natural cytotoxicity receptors (NCRs). They belong to the Ig-superfamily and mediate

tumor and dendritic cell recognition by NK cells (Ferlazzo et al., 2002; Moretta et al., 2001). All NCRs except for NKp44 are expressed on resting and activated NK cells. NKp44, which is solely found in more recently evolved species, is upregulated only upon IL-2 stimulation of specific NK cells (De Maria et al., 2009; Fuchs, Cella, Kondo, & Colonna, 2005; Vitale et al., 2001). Viral hemagglutinins are supposed ligands for NKp46 and NKp44, while other cellular ligands have not been defined. Their existence, however, is demonstrated by the fact that NCR-blocking antibodies are able to abrogate NK cell-mediate lysis of tumor cell (Höglund and Brodin, 2010). Ligands for NKp30 include the nuclear factor HLA-B-associated transcript 3, which can be released from tumor cells, B7-H6 and heparin sulfate proteoglycans, the latter also being a ligand for NKp46 (Bloushtain et al., 2004; Brandt et al., 2009; Pogge von Strandmann et al., 2007). Complementary to NKG2D, NCRs are proposed to be one of the main mediators of NK cell cytotoxicity towards tumor targets (Pende et al., 2009). This is validated by studies showing that the ability of NK cells to lyse tumor targets *in vivo* is diminished by deletion of a single NCR (Halfteck et al., 2009; Pessino et al., 1998; Sivori et al., 1999). Moreover, NKp30 is also associated with interaction between NK cells and dendritic cells (DCs) causing apoptosis and maturation of DCs (Moretta, 2002; Pende et al., 2009).

Within the same family of receptors, the leukocyte immunoglobulin-like receptors (LIRs) can be found, which bind to MHC class I molecules (Colonna et al., 1997). The exact role of LIRs in the regulation of NK cell responses is as of yet unclear, since they act similarly to the more dominant inhibitory KIR and CD94-NKG2A (Navarro et al., 1999). However, the increased affinity of specific LIR receptors to certain antigens could suggest their relevance in NK cell biology (Chapman, Heikeman, & Bjorkman, 1999).

2.1.4 2B4, KLRG1 and co-stimulatory receptors

The functional properties of the 2B4 receptor (CD244) and its ligand CD48 - a surface molecule restricted to the hematopoietic lineage, have so far not clearly been defined (M. H. Brown et al., 1998; Garni-Wagner, Purohit, Mathew, Bennett, & Kumar, 1993; Latchman, McKay, & Reiser, 1998; Valiante & Trinchieri, 1993). Depending on the recruited adapter proteins, the 2B4 receptor can relay an activating or inhibitory signal (Garni-Wagner et al., 1993; K.-M. Lee et al., 2004; McNerney, Lee, & Kumar, 2005). Therefore, it has been postulated that the maturation stage of NK cells as well as the

expressed isoform might influence the outcome when triggering the multi-functional 2B4 receptor (Lanier, 2005; S. O. Mathew, Rao, Kim, Bambard, & Mathew, 2009).

Killer cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor that binds to the classical cadherins, (E-, N- and R-cadherins) (M. Ito et al., 2006; Robbins et al., 2002). As cadherins are commonly expressed on normal, solid tissue, KLRG1-mediated signaling on educated NK cells can prevent damage to healthy tissue (M. Ito et al., 2006). However, apart from setting a threshold for NK cell activation, KLRG1 might also have a role in the missing-self-mediated NK cell response. Consequently, downregulation of E-cadherin, which allows malignant epithelial tumors to metastasize, could in turn also serve as a strategy for NK cells to detect malignant epithelial tissue (Colonna, 2006; Cowin, Rowlands, & Hatsell, 2005; Jeanes, Gottardi, & Yap, 2008).

Co-stimulatory receptors in NK cells are not sufficient to trigger an NK cell response on their own, but provide and alternate mechanism of activation that prevents an attack on normal, healthy tissue. These receptors include DNAM-1, the NKR-P1 receptors and the PILR receptor as discussed hereafter. The DNAM-1 receptor is another member of the Ig-superfamily and is constitutively expressed by roughly half of all NK cells (Shibuya et al., 1996). DNAM-1 acts as an activating co-stimulatory receptor and binds to CD155 and CD112, which can be upregulated on tumor cells (Bottino et al., 2003; Masson et al., 2001; Tahara-Hanaoka et al., 2004). Further evidence suggests that DNAM-1 can act synergistically with other receptors such as 2B4 to mount a cytotoxic immune response independent of conventional activating receptors (de Andrade, Smyth, & Martinet, 2014), thus implying more than just a co-stimulatory function for DNAM-1. In addition, DNAM-1 might play a role in the formation of the immunological synapse, but the precise mechanisms have yet to be elucidated (de Andrade et al., 2014). With the growing importance of DNAM-1, the roles of the two receptors sharing common ligands with DNAM-1; CD96 and TIGIT have also been scrutinized in more detail. CD96, for instance, is able to bind CD155 with a higher affinity than DNAM-1, but although CD96 has an ITIM motif, its biology is poorly understood and it is unclear whether the interaction improves or limits NK cell reactivity (Fuchs, Cella, Giurisato, Shaw, & Colonna, 2004; Meyer et al., 2009; X. Yu et al., 2009). On the other hand, TIGIT has a clear inhibitory function, yet additional *in vivo* data are necessary to determine the potential role of TIGIT in for example autoimmune pathologies where activating signals from DNAM-1 interactions have been described to be involved (de Andrade et al., 2014). As current studies have exemplified the relevance of DNAM-1, TIGIT and CD96 in NK cell

responses, studying them in more detail might reveal valuable insights into pursuing these co-receptors as therapeutic targets.

In humans only one non-polymorphic gene exists for NKR-P1 (CD161), the second co-stimulatory receptor addressed here (Lanier, Chang, & Phillips, 1994). NKR-P1 also belongs to the C-type lectin family and is known in mice for the prototypical NK cell marker NK1.1 (Glimcher, Shen, & Cantor, 1977). In humans NKR-P1 together with NKp80 and NKp65 make up this group of receptors that preferentially bind CLEC2 glycoproteins (Bartel, Bauer, & Steinle, 2013). LLT1, AICL and KACL are the respective ligands of the receptors and are encoded in proximity to their corresponding receptors on the NKRP1 gene (Aldemir et al., 2005; Rosen et al., 2005; Spreu et al., 2010; Welte, Kuttruff, Waldhauer, & Steinle, 2006). All three receptors have very distinct modes of action. While NKR-P1 elicits inhibitory functions when bound to LLT1, NKp80 acts as an activating receptor allowing autonomous control of NK cells that express its ligand AICL (Aldemir et al., 2005; Klimosch, Bartel, Wiemann, & Steinle, 2013; Rosen et al., 2005; Vitale et al., 2001). Further, NKp65 which binds KACL - the CLEC2 protein exclusively expressed on keratinocytes - is thought to facilitate immunosurveillance of human keratinocytes and mount an activating response if necessary (Spreu et al., 2010). Hence, due to their established uniqueness, NKR-P1 receptors might represent a novel mechanism for self/non-self discrimination complementary to MHC-KIR dependent system.

The last co-stimulatory receptor covered here is the paired Ig-like 2 receptor (PILRb) and its isoform PILRa. While the former can associate with DAP-12 and act as an activating receptor, the latter carries an ITIM domain and is regarded as a inhibitory receptor (Mousseau, Banville, L'Abbé, Bouchard, & Shen, 2000; Shiratori, Ogasawara, Saito, Lanier, & Arase, 2004). PILR-L (CD99) is the ligand for these receptors and recognition has been shown to be dependent on the sialylation pattern of the O-linked sugar chains (J. Wang, Shiratori, Satoh, Lanier, & Arase, 2008). Hence, PILRs on NK cells might have a possible role in mediating recognition of carbohydrate chains on target cells, thereby broadening the recognition spectrum of NK cell surveillance.

2.2 Developmental stages of Natural Killer cells

Hematopoiesis is a continuous process that replenishes our blood cells. In humans, hematopoiesis begins in the yolk sac during embryonic development before moving on to the fetal liver and finally ending up in the bone marrow (Fernández & de Alarcón, 2013).

All cells found in the blood originate from one common ancestor, the hematopoietic stem cell (HSC) (LORENZ, UPHOFF, REID, & SHELTON, 1951; Maximow, 1909).

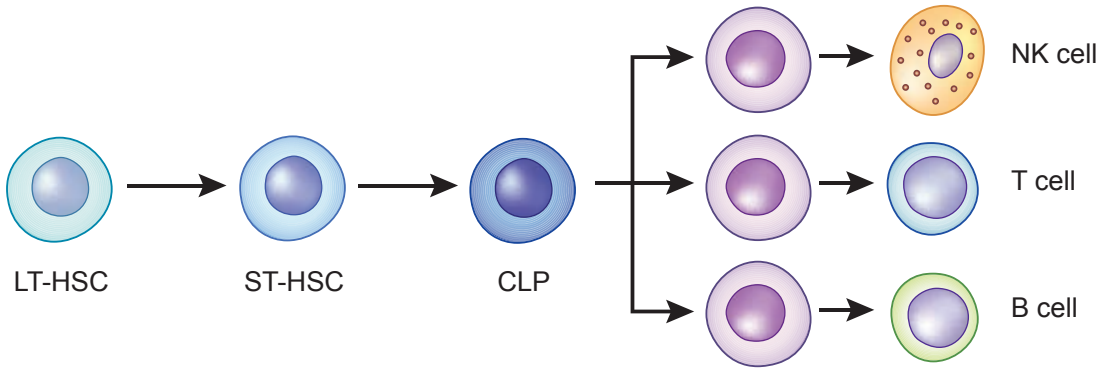

Figure 4. Adapted from a model for hematopoiesis. (Sankaran & Weiss, 2015)

These pluripotent stem cells give rise to daughter cells that are committed to producing multipotent progenitors intended for long-term engraftment and are therefore also called LT-HSCs (Ogawa, 1993). As hematopoiesis progresses, progenitor cells undergo a gradual fate restriction and eventually mature into lineage-committed cells (Figure 4) (Doulatov, Notta, Laurenti, & Dick, 2012). The identity of the numerous hematopoietic cell types is influenced by the expression of transcription and growth factors as well as cytokines that shape the final lineage-specific differentiation (Zhu & Emerson, 2002).

2.2.1 Early development of NK cell precursors

NK cells are part of a broader family of innate lymphoid cells (ILCs), which share a common precursor that gives rise to the phenotypically and functionally distinct ILC1s, ILC2s and ILC3s (Spits et al., 2013). Despite their differences later in development, all ILCs originate from bone marrow-derived $Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ HSCs (Figure 5). As these pluripotent cells start to be committed, their loss of developmental plasticity is evident by the acquisition of CD38 on the surface and they mature into $Lin^{-}CD34^{+}CD38^{+}CD10^{+}$ common lymphoid progenitors (CLPs) In mice, the acquisition of CD122 marks a fate deciding step in NK cell differentiation (Boos, Ramirez, & Kee, 2008).

However, in humans, CD122 expression is virtually undetectable by flow cytometry, thus enforcing the necessity to rely on alternative markers to identify early developmental stages prior to CD94 expression (Freud and Caligiuri, 2006). Some of these markers were recently used to identify a unique cell population that was found to be $CD34^{+}CD38^{+}CD123^{-}CD45RA^{+}CD7^{+}CD10^{+}CD127^{-}$ and thought to represent the first NK lineage progenitor subset (Renoux et al., 2015). In secondary lymphoid organs (SLO), two populations of $Lin^{-}CD94^{-}$ NK precursor can be found in close proximity to their putative progeny: $Lin^{-}CD34^{dim}CD45RA^{+}\alpha_4\beta_7^{bright}CD117^{+}CD161^{+/-}CD94^{-}$ stage 2 and $Lin^{-}CD34^{-}\alpha_4\beta_7^{-}CD117^{+}CD161^{+}CD94^{-}$ stage 3 NK precursors (Figure 5) (Freud and Caligiuri, 2006).

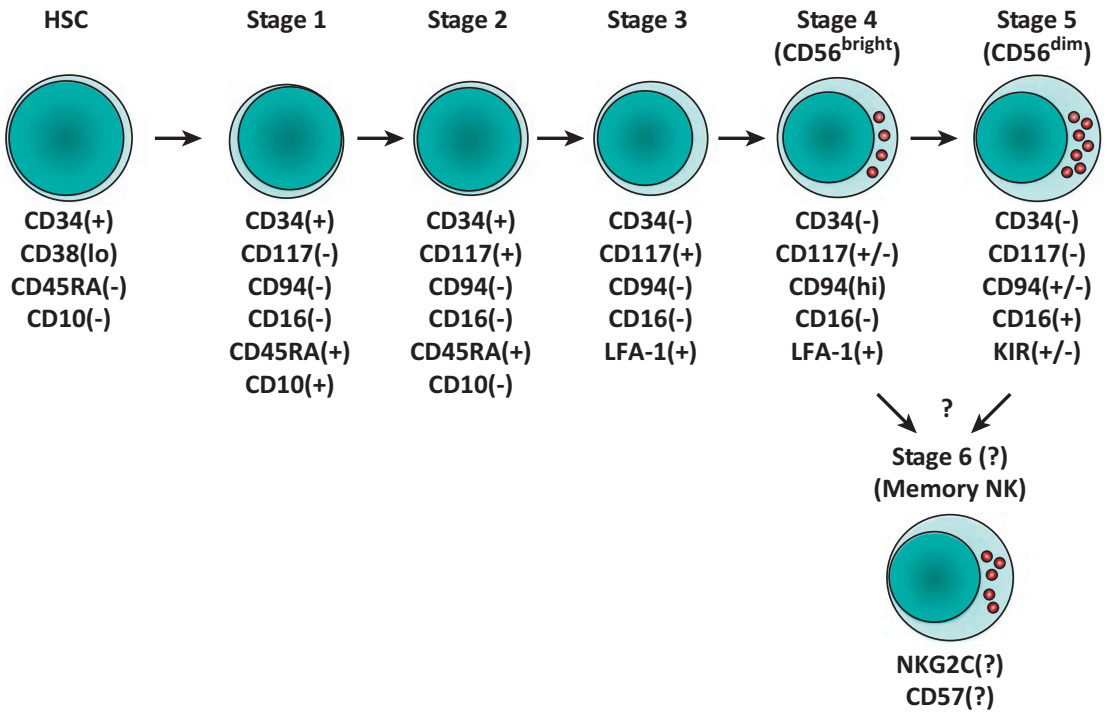

Figure 5. Schematic representation of the cellular intermediates of human natural killer (NK) cell development. (Yu et al., 2013)

Both populations are able to respond to stimulation with interleukin-15 (IL-15) and can give rise to mature NK cells *in vitro*. Yet stage 2 cells clearly represent an earlier point in development, as they are able to differentiate into T cells, DCs and stage 3 NK cells *in vitro* (Freud et al., 2006). In contrast, stage 3 cells are neither able to revert to stage 2 cells nor give rise to T cells or DCs *in vitro*, suggesting that stage 3 cells are the direct progeny of stage 2 cells.

As the NK precursors head towards the final stages of development, they acquire a number of functional receptors in a sequential fashion. First among them is the C-type lectin CD161, followed by CD56, CD94-NKG2A, NKp46, and NKG2D; and completed by the killer immunoglobulin-like receptors (KIRs) and CD16 (Grzywacz et al., 2006; Perussia, Chen, & Loza, 2005). Some of these surface receptors such as CD56, CD117, NKp46, CD94-NKG2A, and the TNF family receptor CD27 are lost again or downregulated as NK cells reach maturity. These expression patterns found *in vitro* coincide with data from clinical samples where neonatal NK cells which are mostly $CD94^{+}KIR^{-}$ show the same progression to $CD94^{-}KIR^{+}$ NK cells in adults (Almeida-Oliveira et al., 2011; Hayhoe, Henson, Akbar, & Palmer, 2010; Sundström et al., 2007).

2.2.2 Late stages of NK cell development: early vs. late differentiation

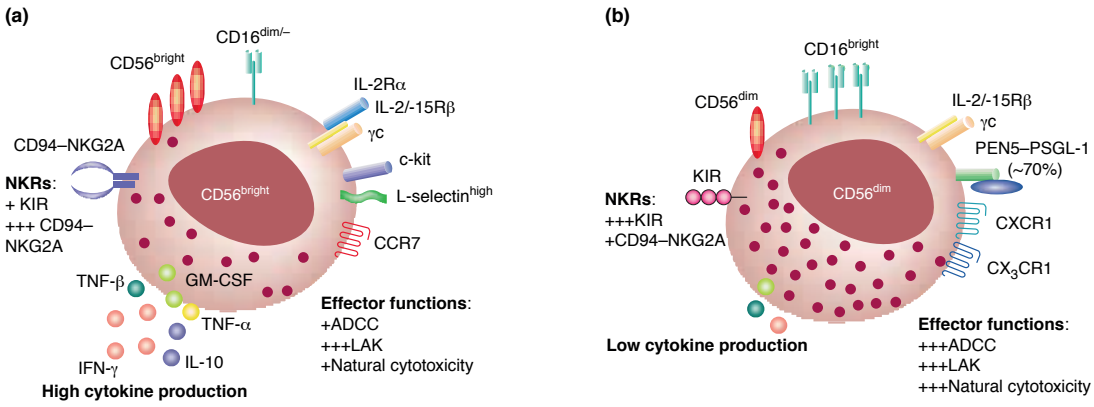

Figure 6. Schema of human natural killer (NK)-cell subsets. (Cooper, Fehniger, & Caligiuri, 2001a)

Mature NK cells are made up of two major subsets, the $CD56^{bright}$ (stage 4) and $CD56^{dim}$ (stage 5) NK cells (Figure 5&6). The $CD56^{bright}$ are classically known as the cytokine-producing subset (Fehniger et al., 2003). They are enriched in SLO and characterized by expression of NKp30, NKp46, DNAM-1, 2B4, LAIR-1, and NKG2D while lacking expression of KIRs, CD16 and perforin in early developmental subsets (Ferlazzo et al., 2004b). Since cytokines produced by NK cells are crucial in early innate host responses, the $CD56^{bright}$ subset plays an important immunoregulatory role during infection. Upon induction by monokines, $CD56^{bright}$ NK cells secrete a trove of factors such as interferon-γ (IFNγ), tumor necrosis factor α/β (TNFα/β), granulocyte macrophage colony-stimulating factor

(GM-CSF), IL-10 and IL-13 (Cooper et al., 2001). In comparison, the $CD56^{dim}CD16^{+}$ NK cells, found in peripheral blood, are proficient at eliciting cytolytic activity (Caligiuri, 2008). For instance, using the abundantly expressed low-affinity Fc-gamma receptor IIIA (CD16A) on their surface $CD56^{dim}$ NK cells are able to bind the constant region of immunoglobulin on the cell surface of opsonized tumor targets. Upon receptor-ligand binding, CD16 mediates a signal which results in degranulation and perforin-dependent target cell lysis, a process also termed antibody-dependent cellular cytotoxicity (ADCC) (Lanier, Ruitenberg, & Phillips, 1988; Perussia, 1998). In addition to the aforementioned pathway, NK cells can efficiently target malignant cells without prior simulation by sensing the absence or loss of major histocompatibility complex (MHC) class I on target cells (Ljunggren & Kärre, 1990). Under normal conditions, NK cell cytotoxicity is averted by signaling through inhibitory KIRs (Thielens, Vivier, & Romagné, 2012). However, MHC class I-deficient patients do not show signs of NK cell-mediated autoimmunity despite the lack of inhibition through HLA-KIR signaling. Thus, in the absence of feedback from inhibitory KIRs, additional mechanisms ensure the regulated immune response of NK cells. This includes binding of HLA-E to its inhibitory ligands to dampen NK cell responses as well as a process termed education, which will be discussed in more detail below. Suffice to say for now that education is key in allowing NK cells to be functionally primed through MHC class I interactions (Anfossi et al., 2006). Accordingly, NK cells which are not primed are unable to kill or lyse target cells that lack MHC class I (Vitale et al., 2002). Additionally, when the dominating inhibitory signal is lost the contribution of lower affinity activating receptors such as NKG2D, NCRs, the nectin and nectin-like receptors and NKp80 need to be taken into consideration (Valés-Gómez, Reyburn, Erskine, López-Botet, & Strominger, 1999) (Bryceson, March, Ljunggren, & Long, 2006). Although both subsets have functionally very distinct properties, it is thought that the $CD56^{bright}$ are precursors to the $CD56^{dim}$ subset and develop in an IL-15-dependent fashion (Huntington et al., 2009). It has also been shown that upon activation with IL-2, NK cells from SLO significantly upregulate CD16, NCRs and expression of KIRs (Ferlazzo et al., 2004b). Further, in the periphery $CD56^{dim}$ NK cells, expressing high levels of CD94, can be found, indicating an intermediate transitional subset from $CD56^{bright}$ to $CD56^{dim}$ NK cells (Jianhua Yu et al., 2010). However, some amount of plasticity remains allowing $CD56^{dim}$ cells to enter back into a $CD56^{bright}$-like phenotype upon stimulation with IL-18 (Vukicevic et al., 2010).

Additional markers have been described that define further steps in the late differentiation of $CD56^{dim}$ NK cells. As $CD56^{dim}$ cells mature, a progressive gain of CD57

and KIRs can be observed which inversely correlates to the levels of NKG2A, and this is associated with reduced NK cell proliferative capacity (Björkström et al., 2010). These changes illustrate a progression from early-differentiated $CD56^{dim}NKG2A^{+}KIR^{-}$ to late-differentiated $CD56^{dim}NKG2A^{-}KIR^{+}$ NK cells (Béziat et al., 2010). Interestingly, both developmental stages have been shown to favorably expand in specific disease settings. For instance, acute infectious mononucleosis (IM), which occurs in adolescents suffering from Epstein-Barr Virus (EBV), is preferentially controlled by $CD56^{dim}NKG2A^{+}KIR^{-}$ NK cells that target lytically replicating B cells (Azzi et al., 2014). These $CD56^{dim}NKG2A^{+}KIR^{-}$ NK cells remain elevated even after viral loads drop below the detection limit hinting at long-term persistence beyond their effector time window (Dunmire et al., 2015). In contrast, acute viral infections from hantavirus, cytomegalovirus (CMV) or chikungunya virus accumulate late-differentiated NK cells manifesting increased expression of the HLA-E binding activating receptor $CD56^{dim}NKG2C^{+}KIR^{+}$ (Béziat, Dalgard, et al., 2011a; Björkström et al., 2011; Lopez-Vergès et al., 2011; Petitdemange et al., 2011). Likewise, this population did not only expand upon infection but was observed to persist over long periods of time, suggesting the existence of a NK cell memory pool.

2.2.3 Immunological memory in innate immunity

Traditionally, the ability to generate an immunological memory was considered a hallmark of the adaptive immune system. However, an increasing amount of evidence suggests that also NK cells are able to mount recall responses. This could therefore be considered the final step in NK cell differentiation. The first evidence of long term persisting NK cells was found in HCMV seropositive patients who retained higher frequencies of NK cells expressing the activating CD94–NKG2C receptor compared to seronegative individuals (Gumá et al., 2004). The concept of actual antigen-specific NK cell memory was later reinforced in mice lacking T and B cells yet these mice were still able to develop a contact hypersensitivity response mediated by NK cells, following sensitization with haptens (O'Leary, Goodarzi, Drayton, & Andrian, 2006). Soon after, the role of ubiquitously resident memory NK cells in the context of a viral infection – MCMV and immunization with virus like particles (VLPs) could also be demonstrated (Paust et al., 2010; J. C. Sun, Beilke, & Lanier, 2009). In humans, it is well established that the CD94-$NKG2C^{+}$ NK cell population, which preferentially expands during acute HCMV infection, can persist as memory NK cells and constitute up to 70% of the total NK cell

population (Foley, Cooley, Verneris, Pitt, et al., 2012b; Lopez-Vergès et al., 2011). Furthermore, in patients that have undergone allogeneic hematopoietic cell transplantation, CD94-NKG2C$^+$ NK cells from HCMV-seropositive donors co-cultured with K562 cells show an increase in the frequency of IFN-gamma$^+$ cells after HCMV reactivation compared to CD94-NKG2C$^+$ NK cells reconstituted from HCMV-seropositive or -seronegative donors in patients without such a reactivation of HCMV. Thus, these data further strengthen the suggestion that a long-lived memory NK cell response exists that is induced by HCMV infection (Foley, Cooley, Verneris, Curtsinger, et al., 2012a). The expansion of NKG2C$^+$ NK cells in HCMV infection itself has been postulated to be mediated by the interaction of the CD94-NKG2C receptor with its ligand HLA-E as well as IL-12 produced by CD14$^+$ monocytes (Figure 7a) (Rölle et al., 2014).

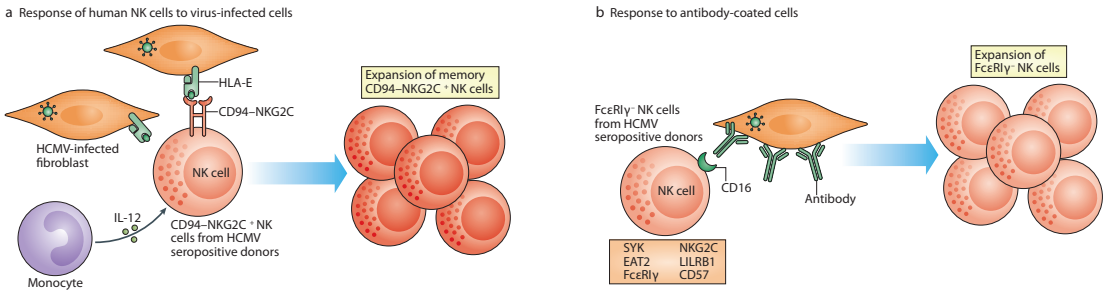

Figure 7. NK cell memory in HCMV-seropositive donors. (Cerwenka & Lanier, 2016)

Yet, the incidence of congenital HCMV infection remained unchanged in individuals that have a null allele for the gene encoding NKG2C compared to controls (Noyola et al., 2012). Therefore, NK cell memory formation, if at all protective in the context of HCMV, is not an NKG2C-specific process but rather reflects the selective, pathogen-specific expansion and persistence of a particularly potent subpopulation. A supporting role of activating KIRs in memory NK cell formation in response to HCMV has been implied by the observation of a specific expansion of KIR2DS2-, KIR2DS4- and KIR3DS1-expressing NK cells, (Béziat et al., 2013). However, it is still unclear which specific factors drive memory formation of NK cells.

A lot of the work so far promoting the idea of memory NK cell formation has been done in the infectious setting of HCMV. However, expansion of CD94–NKG2C$^+$ NK cells has also been observed after infection with other viruses such as hantavirus, HIV and hepatitis C virus (HCV) (Béziat, Dalgard, et al., 2011a; Björkström et al., 2011; Brunetta et

al., 2010), albeit only in individuals who are persistently infected with HCMV. Furthermore an exciting new study described the role of antigen-specific memory NK cells in SIV infected rhesus macaques. Both after infection and vaccination, the formation of a robust antigen-specific memory NK cell population was induced, which specifically targeted dendritic cells pulsed with the major structural (Gag) and envelope (Env) proteins of SIV in an NKG2-dependent fashion (Reeves et al., 2015).

As the ability for cytokines to induce memory in mice had been shown, the possibility was also investigated in humans (Cooper et al., 2009). Similar to the murine studies, NK cells that were briefly exposed to IL-12, IL-15 and IL-18, washed and then cultured in IL-15 for up to 21 additional days, produced highly elevated amounts of IFNγ upon re-stimulation with K562 or IL-12 and IL-15 (Cooper et al., 2009; Romee et al., 2012). In addition, a key role for IL-12 in the expansion of CD94–NKG2C^{+} NK cell subset was also described (Rölle et al., 2014). Both HCMV-driven memory as well as exposure to IL-12, IL-15 and IL-18 results in stable demethylation of the *IFNG* locus (Luetke-Eversloh et al., 2014). This allows NK cells to remember prior exposure to polarizing cytokines even in the absence of a specific antigenic stimulus, thus bestowing them with memory capabilities.

Phenotypically, memory NK cells have been mainly characterized in the context of HCMV infection. NK cells from HCMV-seropositive donors typically express high levels of the CD94-NKG2C in conjunction with CD57, and lack NKG2A (Lopez-Vergès et al., 2011). In addition, as mentioned above, the expression of specific activating KIRs such as KIR2DS2, KIR2DS4 or KIR3DS1 can be observed (Béziat et al., 2013). Two recent back-to-back studies defined the genetic and epigenetic changes in NK cells from HCMV-seropositive individuals in more detail. They found that so-called 'adaptive NK cells' are characterized by a lack of high-affinity IgE receptor subunit-γ (FcεRIγ), variable loss of the tyrosine kinase SYK and transcription factor EWS/FLI1-activated transcript 2 (EAT2) as well as downregulation of the transcription factor pro-myelocytic leukemia zinc finger (PLZF) (Lee et al., 2015; Schlums et al., 2015). Interestingly, both the CD94-NKG2C^{-} and CD94-NKG2C^{+} NK cells contained a FcεRIγ-deficient subset, potentially implicating the FcεRI in shaping NK cell memory subsets which exhibit elevated CD16-mediated responses against HCMV-infected target cells coated with HCMV-specific antibodies (Figure 7b) (Lee et al., 2015).

The apparent existence of a memory NK cell compartment poses the obvious question of the functional relevance of this population. Contrary to what one might

expect, CD94-NKG2C$^+$ NK cells detected in HCMV-seropositive individuals display a reduced response to inflammatory cytokines such as IL-12 and IL-18 (Béziat et al., 2013). Moreover, upon re-activation with pertussis or H1N1 influenza vaccine antigens, lower levels of IFNγ production and degranulation were observed (Nielsen et al., 2015). This observation that HCMV-expanded CD94-NKG2C$^+$ cells have diminished responses to heterologous challenges could be highly relevant for designing vaccines against viruses other than HCMV and needs to be dissected in more detail in future studies.

2.3 Education and Disarming of NK cells

As mentioned above, since KIRs separate with great diversity and stochastic distribution, nearly every differentiated NK cell expresses at least one self-specific KIR (Andersson, Malmberg, & Malmberg, 2010; Uhrberg et al., 1997). Yet around 10% of NK cells do not express any self-specific KIR at all, posing the question what prevents these cells from becoming autoreactive (Anfossi et al., 2006).

Currently it is known that engagement of inhibitory receptors on NK cells by self-MHC class I molecules is necessary to functionally prime NK cells to recognize missing-self, a process termed education or licensing (Kim et al., 2005; 2006). In situations where self-MHC class I ligands are not available, NK cells become hyporesponsive and are no longer able to secrete cytokines or mediate cytotoxicity upon stimulation (Fauriat et al., 2008). Accordingly, NK cells that develop in the absence of MHC class I are unable to kill MHC class I-deficient tumor cells and fail to reject hematopoietic bone marrow grafts *in vivo* (Fernandez et al., 2005; Kim et al., 2005; 2006). Still, in the absence of KIR-MHC-mediated education, NK cell receptors that recognize ligands other than MHC class I are able to compensate for the lack of self-MHC class I recognition. The most prominent example for this is NKG2A, which can bind HLA-E and elicit NK cell function of KIR-negative NK cells, thus acting complementary to KIR-mediated education (Yu et al., 2007). The models which explain how NK cell education is mediated through KIR-MHC interaction, are summarized below and in Figure 8:

Arming

The arming model proposes the requirement of inhibitory receptors to bind NK cells in order to mediate functional competence (Raulet & Vance, 2006). Thus in the absence of self-MHC class I specific inhibitory receptors, NK cells remain functionally incompetent. This would however imply an instructive role of the inhibitory receptors, actively promoting functional maturity of NK cells. As the inhibitory receptors of NK cells customarily do not mediate developmental signals but rather immunological stimuli the validity of this model has yet to be proven (Goodridge et al., 2015).

Disarming

In contrast, the disarming model postulates that NK cells which lack inhibitory receptors for self-MHC class I, become hyporesponsive due to constant activating stimuli (Raulet & Vance, 2006). This model might best be described as mediating a form of NK anergy to ensure self-tolerance. Thus when self-MHC class I molecules are available for inhibitory receptors to interact with, NK cells are not prompted to become anergic and develop into fully functional and reactive NK cells. Compared to the arming model, this means that NK cells are initially responsive and their environment makes them hyporesponsive, while the arming model suggests that NK cells are constitutively hyporesponsive. Several studies have been done which support the disarming model for education. For instance NKG2D ligand transgenic mice have been shown to foster development of an entirely hypofunctional NK cell compartment (Oppenheim et al., 2005; K. Wiemann et al., 2005). Furthermore, in humans NK cells expressing the activating KIR2DS1 are hyporesponsive when derived from donors that express the cognate ligand HLA-C2, backing the observations made in mice (Fauriat, Ivarsson, Ljunggren, Malmberg, & Michaëlsson, 2010). Together, these data suggest that the constant presence of activating ligands leads to anergy in NK cell subsets that express the corresponding activating receptor (Höglund and Brodin, 2010).

***Cis*-interaction**

The *cis*-interaction model is rooted in the observation that NK cell inhibitory receptors in mice are able to bind MHC class I in *cis* on the same cell (Chalifour et al., 2009). Murine inhibitory receptors are able to transmit inhibitory signals despite the lack of ligands in trans. These so called ‘unengaged‘ receptors prevent NK cells from efficiently engaging their targets leading to a hyporesponsive phenotype. However, inhibitory receptors on murine NK cells can bind their ligands in *cis*, which sequesters them away from the interaction site and allows engagement of the target. Thus *cis*-interactions counteract ‘unengaged’ receptors by blocking inhibitory receptors and enabling interaction of

activating receptors, which leads to improved responsiveness of NK cells (Chalifour et al., 2009). Experiments where a truncated version of the inhibitory receptor was introduced into mice, further elucidated the role of *cis*-interactions during NK cell education. As the truncated inhibitory receptor was no longer able to interact with its ligand in *cis*, NK cells were not educated. However, the truncation did not effect engagement of ligands in trans or inhibitory signaling (Bessoles et al., 2013). Thus underlining the importance of *cis*-interactions for NK cell education.

Rheostat

The rheostat model suggests that the process of NK cell education is fluid rather than a rigid all or nothing response, allowing NK cells to adapt their response to changes in the environment. This idea is supported by evidence showing that NK cell responsiveness is dependent on the strength of the inhibitory signal it encounters (Brodin, Kärre, & Höglund, 2009; Johansson et al., 2005). For instance, NK cells that express multiple inhibitory receptors for self-MHC class I respond in an almost additive fashion more frequently with boosted effector functions (Brodin et al., 2009; Joncker, Fernandez, Treiner, Vivier, & Raulet, 2009). As such, the affinity of the inhibitory receptor to its MHC class I ligand can also influence the NK cell educational process (Jonsson, Yang, Kim, Taffner, & Yokoyama, 2010). NK cells would therefore be able to 'tune-up' or 'tune-down' their responsiveness in a quantitative manner depending on the inhibitory signal they receive (Brodin et al., 2009; Joncker et al., 2009). Hence, the rheostat model does not claim exclusiveness, but rather goes hand-in-hand with the arming and disarming models (Brodin & Höglund, 2008).

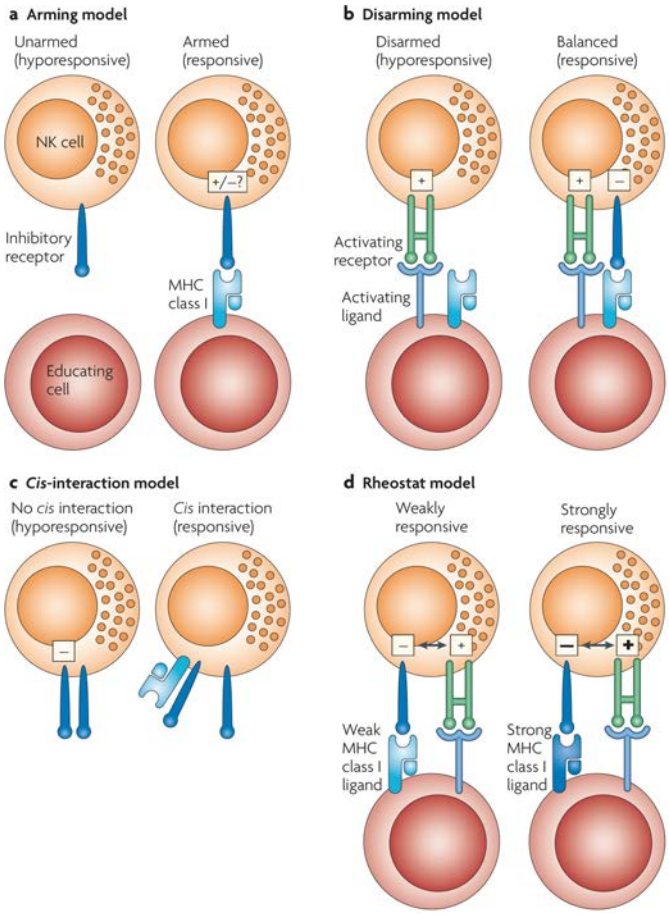

Figure 8. Models for NK cell education. (Höglund and Brodin, 2010)

Importantly, it needs to be noted that NK cell education is not static and can adapt rapidly to changes in the environment (Pradeu et al., 2013). In fact, when educated NK cells are adoptively transferred to MHC class I-deficient hosts, they become hyporesponsive within a matter of days (Joncker et al., 2010). Inversely, when hyporesponsive NK cells are transferred to an educating MHC environment it leads to uptuning of their functionality (Joncker et al., 2010).

All so-called 'hyporesponsive' NK cells are effectively non-educated. Interestingly, a protective function during viral infection has been attributed to non-educated NK cells. During the primary response to MCMV infection, they dominate the reactive NK cell population, efficiently clearing viral infection in the host independent of cognate activating receptor engagement (Orr et al., 2010). Also, upon infection with Listeria monocytogenes, non-educated NK cells are able to respond by secreting IFN-γ, demonstrating that their lack of response in terms of education was not due to a global defect in NK cell function (Fernandez et al., 2005). Moreover, cytokine stimulation with

IL-2 or IL-12 and IL-18 likewise induces an effector phenotype in (self-) receptor negative NK cells, suggesting that given sufficient stimulation, NK cells can reach a cytolytic phenotype in the absence of any educating input (Björkström et al., 2010). Collectively, these findings reveal that NK cell tolerance or anergy is reversible and that functionality of non-educated or disarmed NK cells can be restored.

Although the current consensus is that education is important for the functionality of NK cells, its specific role for instance during the acute phase of a viral infection is still poorly understood. Our own data as well as a study using a yellow fever vaccine (YFV 17D) to model viral infections suggest that educated and non-educated NK cells both respond to a similar degree to acute viral infection (Marquardt et al., 2015). Furthermore, researchers were able to show that the response to YFV 17D was mainly mediated by early-differentiated NK cells, indicating that differentiation but not education seems to be central in the response to vaccination. Hence, dissecting the function of education in NK cell responses may help to better understand and tune NK cell-targeted vaccination and therapies.

2.4 The NK cell immunological synapse

As outlined in the previous chapters, ligand-receptor interactions are key in controlling NK cell biology. Therefore, the mechanisms which facilitate an efficient cross-talk between NK cells and other leukocytes or transformed cells have been studied with great interest. NK cells are described to form three different types of immunological synapses (IS) - inhibitory, cytotoxic and regulatory (Barreira da Silva and Münz, 2011). Each of these interactions is characterized by a set of molecules, which interact at the interface between the NK cell and the target cell. The formation of the IS begins with the initiation stage, during which primary contact between the NK cell and the putative target is established through binding of integrins, such as LFA-1 and ICAM-1. While integrins gather at the periphery of the synapse, receptors accumulate in the center (Orange et al., 2003). At this point, the fate of the synapse is decided by whether the majority of receptors are activating or inhibitory, thus defining the dominant signal. Accumulation of inhibitory KIRs and HLA molecules determine an inhibitory outcome for the IS, and after a transient interaction between the NK cell and the putative target, the NK cell moves on (Davis et al., 1999).

However, if activating signals prevail, then the formation of a long-lived cytotoxic synapse is initiated. This includes the accumulation of molecules in distinct regions of the IS, thus creating a so called supramolecular activation cluster (SMAC) that is divided into a central and a peripheral zone (cSMAC and pSMAC) (Figure 9) (Orange, 2008). As a first step, actin reorganizes and polarizes to the cytotoxic IS, supporting clustering of the receptors. Following this, lytic granules start moving along microtubules to the microtubule-organizing center (MTOC), which itself begins to polarize towards the IS. Once the MTOC has reached the IS, the actin network starts to disassemble in spots in order to create channels that will allow the lytic granules to gain access to the plasma membrane. With the help of myosin IIA, lytic granules are able to traverse the acting network and fuse with the plasma membrane, subsequently releasing their content, such as perforin or granzyme into the synaptic cleft (D. Liu et al., 2005; Sanborn et al., 2009). In the final stages of the formation of the IS, actin reorganization is likely to facilitate NK cell detachment from the target cell (Burshtyn, Shin, Stebbins, & Long, 2000). Furthermore, after completion, NK cells are able to rapidly restore their cytotoxic potential by regenerating lytic granules giving the potential of 'serial killing' targets (Bhat & Watzl, 2007; Merrill, Ullberg, & Jondal, 1981). Thus, the cytotoxic synapse serves to concentrate lytic effector molecules at the synapse while protecting neighboring cells from exposure to these detrimental molecules, enabling efficient and specific killing of multiple subsequent targets (Orange, 2008).

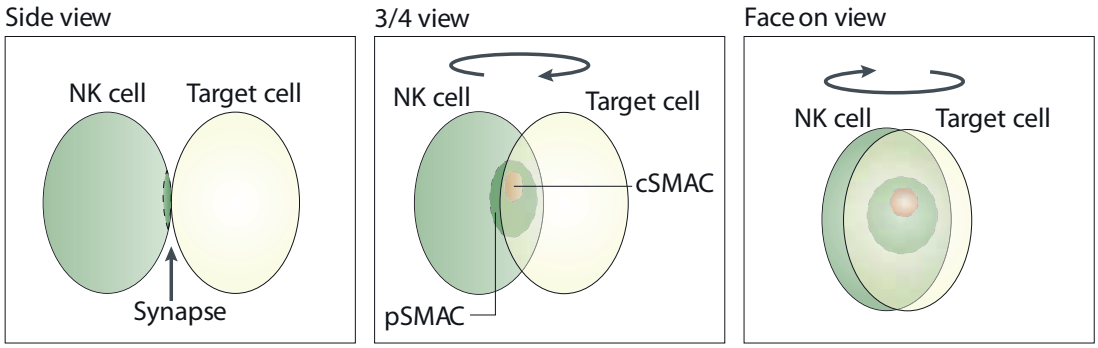

Figure 9. Adapted from the prototypical mature NK cell lytic synapse. (Orange, 2008)

The third type of synapse which is formed by NK cells is the regulatory synapse and its function is related to NK cell priming by myeloid APCs (Ferlazzo et al., 2002; Lucas et al., 2007). Although DCs are able to activate NK cells solely via soluble cytokines, cell

contact can be important for increased transmission efficiency of certain signaling molecules (Barreira da Silva and Münz, 2011). Interestingly, mature DCs are able to mediate activation of resting NK cells via the regulatory synapse, while simultaneously preventing themselves from being killed (Brilot et al., 2007; Ferlazzo et al., 2002). NK cells need the cross-talk with DCs to release cytokines, increase their cytotoxic potential, survive and proliferate (Brilot et al., 2007; Strowig et al., 2008). In parallel, the accumulation of MHC class I expression on mature DCs represses NK cell cytotoxicity through inhibitory receptor signaling (Ferlazzo et al., 2002). Furthermore, expression of the protease inhibitor 9 (PI9) in DCs helps to inhibit granzyme B-mediated apoptosis (Hirst et al., 2003). An in-depth study of the regulatory synapse showed that the actin-dependent stabilization of MHC class I molecules at the IS is essential to DC survival. Likewise, maturation of the regulatory synapse is associated with an accumulation of filamentous actin (f-actin) at the IS (Barreira da Silva et al., 2011). Recruitment of inhibitory KIRs and CD94-NKG2 receptors as well as IL-15 receptor components to the IS happens rapidly. Thus, activating and inhibitory receptors find themselves at the same spot at same time. Segregation into separate domains at the center of the IS seems to be the key to making signaling of both a stimulatory and inhibitory nature possible in parallel (Brilot et al., 2007). As maturation of the regulatory synapse progresses, more signals emerge such as IL-12, mediating IFN-γ secretion by NK cells ("NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs.," 2004a). Thus, stabilization of the KIR-HLA interaction through f-actin accumulation allows for efficient priming of NK cells without jeopardizing the DC.

PART III – DENDRITIC CELLS

Dendritic cells are prototypic antigen presenting cells (APCs) that express MHC class I and II and are adapted to processing and presenting peptide fragments to cells of the innate and adaptive immune system. Accordingly, DCs are constantly patrolling their environment in search of pathogen and/or stress signals ("Dendritic cells and the control of immunity.," 1998).

2.1 Dendritic cell subsets

The different types of DCs are categorized into preDCs, conventional DCs and inflammatory DCs (Figure 10). Pre-DCs are defined by their lack of dendrites and classical DC function in steady state and include plasmacytoid DCs and monocytes ("IPC: Professional Type 1 Interferon-Producing Cells and Plasmacytoid Dendritic Cell Precursors," 2005). All subset are uniquely defined by the expression of specific surface markers, localization and pattern recognition receptors (PRRs). These PRRs allow DCs and other innate cells to recognize pathogen and danger associated molecular patterns (PAMPs and DAMPs) which are distinctive for microbial pathogens and damaged or apoptotic cells, respectively ("Pattern recognition receptors and inflammation.," 2010). There are a lot of different kinds of PRRs described that mediate recognition of specific PAMPs and DAMPs, but only members of the toll-like receptor (TLR) family will be discussed in more detail below.

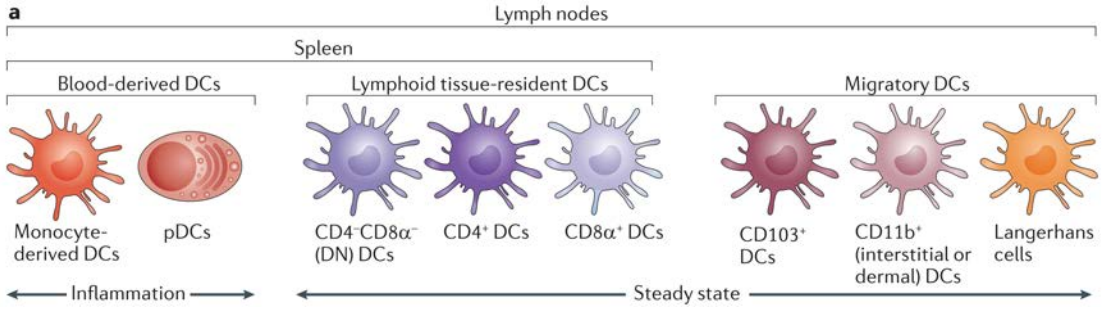

Figure 10. Organization of the dendritic cell network. ("Transcriptional programming of the dendritic cell network.," 2012)

Plasmacytoid DCs (pDCs) are found throughout the body and are an important source of type I interferons upon viral infection ("A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses.," 1985; "Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation.," 1978). In humans they are characterized by BDCA-2 and BDCA-4 expression and are considered poor antigen presenters. Instead, pDCs are able to recognize RNA as well as unmethylated DNA motives consisting of cytosine and guanine nucleotides linked by a single phosphate (CpG

motives) on bacterial and viral genomes via TLR7 and TLR9 ("BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood.," 2000; "DNA activates human immune cells through a CpG sequence-dependent manner.," 1999; "Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets.," 2002; "Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system?," 2011; "Regulation of Toll-like receptors in human monocytes and dendritic cells.," 2001). Both these TLR receptors are abundantly expressed in pDCs and upon induction lead to secretion of IFN-α and IFN-β which in turn stimulates NK cell cytotoxicity (Gerosa et al., 2005). In addition, pDCs are also an important source of IL-12 for NK cell activation during MCMV infection ("Cross-talk between dendritic cells and natural killer cells in viral infection.," 2005; "Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity.," 2005).

In contrast, conventional DCs (cDCs) are specialized for antigen presentation and are further divided into migratory DCs and lymphoid tissue-resident DCs based on their localization in tissue. Migratory DCs develop in peripheral tissue, where they act as sentinels, constantly sampling their surroundings and then migrate to draining lymph nodes ("Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes.," 2008; "Origin and development of dendritic cells.," 2010). Langerhans cells, dermal DCs as well as several subsets such as BDCA-1$^+$ DCs, CD141$^+$ DCs and CD16$^+$ DCs are all part of the migratory DCs ("Epidermal Langerhans cells--changing views on their function in vivo.," 2006; "Human and murine dermis contain dendritic cells. Isolation by means of a novel method and phenotypical and functional characterization.," 1993; "Review of human DC subtypes.," 2010). The second category of lymphoid tissue-resident DCs can be found in murine spleen, thymus and lymph nodes and are further classified by the expression of CD4 and CD8α ("CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen.," 2000). Of note, the murine CD8α$^+$ DCs which are best known for their ability to cross-present antigens and hence prime CD8$^+$ T cells are also part of this family ("CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo.," 2000). Unlike migratory DCs, lymphoid tissue-resident DCs do not traffic at all but develop from precursor in the lymphoid tissue itself ("Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes.," 2006). cDCs can sense bacteria, virus and fungi through the TLR1, 2, 3, 5, 6, and 8 upon which they produce IL-12, TNF and IL-6 amongst others ("Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial

antigens.," 2001a; "Toll-like receptors and dendritic cells: for whom the bug tolls.," 2004a).

The last category of DCs are the inflammatory DCs that only differentiate from precursors upon infection or stress signals. Their TLR repertoire is similar to that of cDCs but these cells can in addition respond to LPS due to their expression of TLR4 ("Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens.," 2001b; "Toll-like receptors and dendritic cells: for whom the bug tolls.," 2004b).

2.2 Cross-talk between DCs and NK cells

2.2.1 NK cell priming by DCs

NK cell-DC interactions take place in SLOs and sites of inflammation, which is also the location where the $CD56^{bright}$ subset is enriched (Ferlazzo et al., 2004b; 2004a). Maturation of NK cells upregulates chemokine receptors on their surface, rendering them more efficient at migrating to SLOs to which mature DCs home to, facilitating their interaction at these sites (Fehniger et al., 2003). In addition, a number of secreted cytokines are also important for NK cell activation. For instance, type I IFNs trigger NK cytotoxicity and control IL-12 production by cDCs, which in turn modulates IFN-γ expression by NK cells ("Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo.," 2002). Together with IL-18, IL-12 promotes IFN-γ secretion by NK cells in response to EBV, influenza, parasites and bacteria (Strowig et al., 2008). Another important cytokine for NK cell biology is IL-15, which has been implicated in the development, survival and proliferation of these innate lymphocytes (Brilot et al., 2007; Huntington et al., 2007). Among DCs, a subset of migratory DCs found in the skin called Langerhans cells expresses especially high levels of IL-15 and IL-15Rα, which gets trans-presented to NK cells expressing the IL-15Rβ and γ chains to promote survival and proliferation of NK cells ("Mature myeloid dendritic cell subsets have distinct roles for activation and viability of circulating human natural killer cells.," 2005a). However, although DCs activate NK cells primarily via cytokines to initiate cytokine production by NK cells and increase their cytotoxic activity, cell contact can be important sometimes to ensure efficient NK cell

activation, thus emphasizing the potential necessity of the regulatory synapse (Brilot et al., 2007; Strowig et al., 2008).

2.2.2 DC editing by NK cells

NK cells are primed by DCs and inversely are responsible for modulating the DC population to ensure efficient antigen presentation, a process termed DC editing (Moretta, 2002). Despite their expression of MHC class I, NK cells are able to lyse myeloid cells due to an abundance of activating ligands that overcome the inhibitory signal. The main targets of DC editing are non-activated microglia cells, TLR-activated macrophages and immature DCs (Ferlazzo et al., 2003). Interestingly, the frequency of cells plays a role in NK cell-mediated killing of DCs. When activated NK cells are present in large numbers, they competently kill immature DCs via NKp30, NKp46 and the co-receptor DNAM-1 ("Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction.," 2006; "NK cell-mediated lysis of autologous antigen-presenting cells is triggered by the engagement of the phosphatidylinositol 3-kinase upon ligation of the natural cytotoxicity receptors NKp30 and NKp46.," 2001) while at low ratios, NK cells seem to promote DC maturation and survival, mediated by IFN-γ and TNF ("Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells.," 2002). As soon as DCs start maturing, they upregulate HLA-E, which consequently protects them from NK lysis (Ferlazzo et al., 2002).

PART IV – EPSTEIN BARR VIRUS

2.1 EBV life cycle

Epstein-Barr virus (EBV) is one of eight human herpesviruses and was discovered more than fifty years ago as the first human tumor virus ("MORPHOLOGICAL AND BIOLOGICAL STUDIES ON A VIRUS IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA.," 1965; "VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S

LYMPHOMA.," 1964). It is part of the *gamma-herpesviridae* subfamily, which is known to infect only simians and has a long history of coevolution with our species ("Epstein Barr Virus Volume 2," 2015a). Similar to other herpesviruses, EBV is oromucosally transmitted via saliva exchange and persistently infects over 95% of adults (Cesarman, 2014) ("Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory.," 2014). EBV was found to be highly but not exclusively B-lymphotropic and has potent B lymphocyte growth-transforming abilities, enabling it to persist in B cells and induce a number of B cell malignancies. The most common disease associated with EBV is infectious mononucleosis (IM), the symptomatic manifestation of acute primary infection (Balfour et al., 2013; Luzuriaga and Sullivan, 2010). Furthermore, the association between EBV and certain malignancies is very well established, such as Burkitt lymphoma, classical Hodgkin lymphoma, post-transplant lymphoproliferative disease and nasopharyngeal carcinoma ("Co-infections, inflammation and oncogenesis: future directions for EBV research.," 2014; "Spectrum of Epstein-Barr virus-associated diseases.," 2006b). Additionally in recent years, EBV has also been implicated in some autoimmune conditions such as multiple sclerosis ("Epstein-barr virus infection and multiple sclerosis: a review.," 2010).

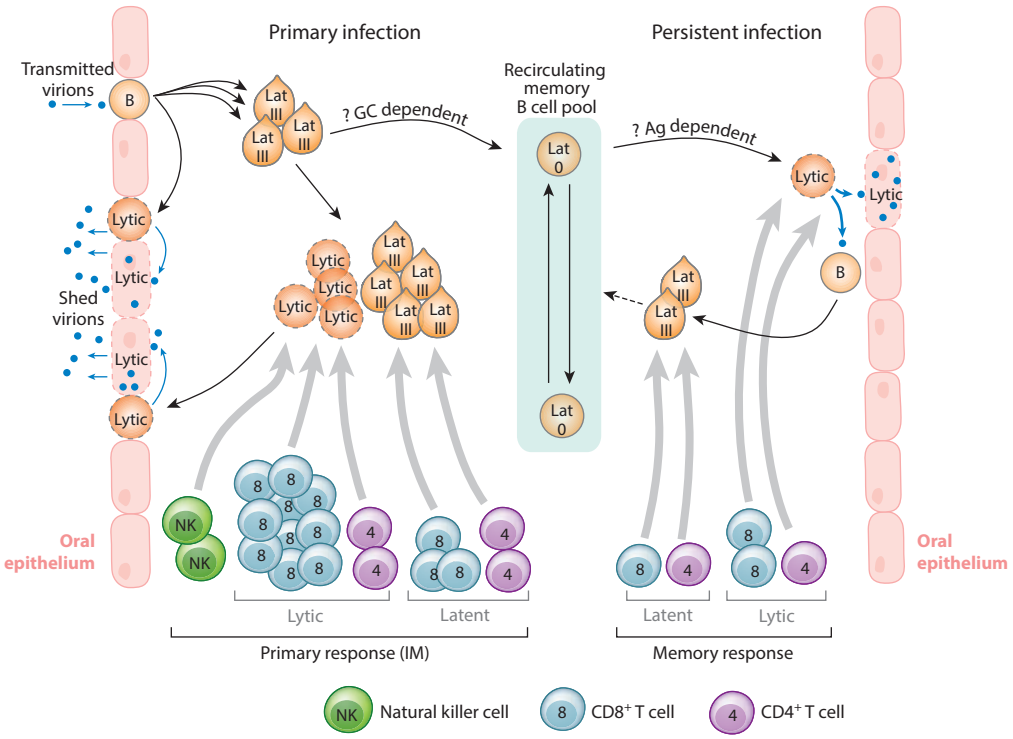

Figure 11. A schematic view of EBV infection and persistence in the immunocompetent host. (Taylor et al., 2015)

Primary infection with EBV leads to initial lytic replication in submucosal B cells and epithelial cells of the oropharynx (Figure 11) (Taylor et al., 2015). At this point, EBV infected individuals are producing vast amounts of virions and are highly contagious. Once EBV enters the next stage of its life cycle, it no longer produces virions and persists in one of the four stages of latency that are characterized by specific expression patterns of viral genes (Table 2) ("EBV gene expression and regulation," 2007). This is also when the infection spreads through proliferation of latently infected B cells. These virus-carrying B cells express six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and -LP), two latent membrane proteins (LMP1 and 2), two EBV encoded RNAs (EBERs) and various microRNAs, which drive Latency III infection (Cesarman, 2014). Subsequent down-regulation of the growth-transforming program allows B cells to enter Latency 0. In this state, EBV is able to persist as a truly latent infection in the B cell memory pool throughout the host's life. Occasionally, reactivation of EBV into lytic replication can occur in plasma cells, leading to virus shedding in the throat ("The pathogenesis of Epstein-Barr virus persistent infection.," 2013). Lytic replication has been shown to be associated with the expression of most virally encoded genes, mediating amplification of the viral genome, synthesis of viral structure proteins, formation of the viral capsid and production of new infectious virions which can be spread to other individuals ("Epstein Barr Virus Volume 2," 2015b). Integral to lytic cycle induction are the virally encoded transcription factors BZLF1 and BRLF1, which are sufficient to induce lytic replication in latently infected cells ("Lytic cycle switches of oncogenic human gammaherpesviruses.," 2007).

Latency program	**Expressed viral genes**
Latency 0	None
Latency I	LMP-2A/EBNA-1
Latency II	EBNA-1, LMP-1, LMP-2A, -2B
Latency III	EBNA-1, -2, -3, -4, -5, -6, LMP-1, LMP-2A, -2B, EBERs

Table 2. EBV latency programs ("Epstein-Barr virus oncogenesis and the ubiquitin-proteasome system.," 2004)

2.2 Immune response to EBV

Disease progression of EBV has been hard to study, since early primary infection is asymptomatic and therefore difficult to detect. Accordingly, much of the knowledge on the immunology of EBV was collected in IM patients after onset of symptoms. Only recently a prospective study that followed college students to monitor the immunological changes before and during onset of IM provided some novel insights into the early immune response to primary EBV infection. For instance, virus was not detected directly after transmission in the oral cavity but only shortly before onset of symptoms and while the number pDCs was decreased during the incubation period, NK cells only came up after disease onset (Dunmire et al., 2015). In addition, the lack of a murine-tropic equivalent of EBV meant that no small animal model was available to study the virus *in vivo*, until the development of mice reconstituted with a human immune system (see next chapter) ("Development of a human adaptive immune system in cord blood cell-transplanted mice.," 2004a). Accordingly, EBV was the first human pathogen to be studied in this novel mouse model. Although many unresolved issues remain the currently defined key players of the innate and adaptive immune response are outlined below.

2.2.1 Innate

The first responders to acute EBV infection might possibly be NK cells, which undergo a significant expansion in peripheral blood (Balfour et al., 2013; Williams et al., 2005). Humanized mice challenged with EBV increase an early subset of NK cells that is predominantly KIR-negative and able to respond prior to $CD8^+$ T cells. Furthermore, when NK cells were depleted in mice, the lack of the NK cell response led to elevated $CD8^+$ T cell numbers and an exacerbated disease progression (Chijioke et al., 2013). Interestingly, a similar subset of $NKG2A^+KIR^-$ NK cells was also found to be more frequent in toddlers and young children compared to adolescents or adults and this NK cell subset could efficiently degranulate and proliferate in response to lytic EBV-replicating B cells. Thus, a decrease in the frequency and number of these early-differentiated NK cells during the first decade of life might probably causally be associated to the increase in severity of illness and the symptomatic $CD8^+$ T cell lymphocytosis found in adolescents suffering from IM (Azzi et al., 2014). In addition, $CD56^{bright}$ NK cells secreting IFN-γ were

found to prevent B cell transformation by EBV *in vitro* (Strowig et al., 2008). Together, these observations show that NK cells are vital in limiting primary lytic EBV infection.

Other responders of the innate immune system include the myeloid compartment. Using their TLRs, monocytes and pDCs are able to sense virions and/or un-methylated EBV genomes, respectively ("Epstein-Barr virus induces MCP-1 secretion by human monocytes via TLR2.," 2007; "Epstein-Barr virus promotes interferon-alpha production by plasmacytoid dendritic cells.," 2010; "TLR9 contributes to the recognition of EBV by primary monocytes and plasmacytoid dendritic cells.," 2010). Furthermore, conventional DCs are able to detect EBERs released from latently infected cells ("Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3.," 2009). Hence, both the antiviral type I IFNs secreted by pDCs as well as IL-12 secreted by cDCs support and activate NK cells responses (Strowig et al., 2008).

2.2.2 Adaptive

The adaptive immune response to EBV, particularly in IM, is characterized by a large expansion of the $CD8^+$ T cell compartment, with up to 50% of the $CD8^+$ T cell population typically responding to early lytic epitopes ("CD8+ T cell responses to lytic EBV infection: late antigen specificities as subdominant components of the total response.," 2013; "Cellular responses to viral infection in humans: lessons from Epstein-Barr virus.," 2007a). As lytic infection progresses, immune evasion proteins downregulate antigen presentation on cells, thus reducing their availability to $CD8^+$ T cells ("Cooperation between Epstein-Barr virus immune evasion proteins spreads protection from CD8+ T cell recognition across all three phases of the lytic cycle.," 2014; "Immune responses to Epstein-Barr virus: molecular interactions in the virus evasion of CD8+ T cell immunity.," 2010). Responses to the ensuing latent program are only mounted by 5% of $CD8^+$ T cells and mainly focused on the EBNA3 protein family that account for 60% of latent genes ("Cellular responses to viral infection in humans: lessons from Epstein-Barr virus.," 2007b; "Tonsillar homing of Epstein-Barr virus-specific CD8+ T cells and the virus-host balance.," 2005). As CD8 expansion occurs simultaneously with onset of symptoms, the hypothesis that IM is an immunopathologic disease is further reinforced (Balfour et al., 2013). Finally, as the viral loads drop, contraction of the peripheral $CD8^+$ T cell

compartment back to steady state levels also takes place, indicating a resolution of EBV infection (Taylor et al., 2015).

In contrast, $CD4^+$ T cell responses remain small and are mainly directed at the latent antigens of EBV (Balfour et al., 2013). Especially EBNA1-specific $CD4^+$ T cells can be found at high frequencies in healthy virus carriers, but not during IM ("Human CD4(+) T lymphocytes consistently respond to the latent Epstein-Barr virus nuclear antigen EBNA1.," 2000). As EBNA1 presentation relies on a mechanism that first degrades and processes the antigen, its accessibility to APCs is delayed. This could reflect the postponed response of $CD4^+$ T cells and the ensuing antibody response ("Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection.," 1987a; "Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display.," 2010).

B cells, which albeit being the primary target, are still able to secret a wide range of antibodies in response to lytic and latent cycle antigens constitute the third pillar of the adaptive response. The presence of IgM and rising IgG titers toward the virus capsid antigen complex as well as absence of EBNA1-specific antibodies are used as serological tools in the diagnosis of IM. ("Performance of the architect EBV antibody panel for determination of Epstein-Barr virus infection stage in immunocompetent adolescents and young adults with clinical suspicion of infectious mononucleosis.," 2014). While the EBNA1 response is delayed and is maintained at a stable level only after 3-6 months, IgG antibodies against EBNA2 peak soon after disease onset before they start to decline again. Healthy virus carriers eventually present a serological profile that includes antibodies against the viral capsid and EBNA1 ("Antibody responses to Epstein-Barr virus-determined nuclear antigen (EBNA)-1 and EBNA-2 in acute and chronic Epstein-Barr virus infection.," 1987b).

PART V – HUMANIZED MICE

Humanized mice (huMice) have become a highly valued tool to study processes of the human immune system that can otherwise not be modeled in healthy donors or patients. To generate these models, mouse strains that lack mouse lymphocytes and whose myeloid compartment tolerates human cells are currently used (Leung et al., 2013).

Preferentially, the non-obese diabetic (NOD) or the BALB/c mouse strain with *scid* or RAG mutations are used (Ishikawa et al., 2005) ("Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells.," 2005a; "Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice.," 2011a). The *scid* mutation or absence of one of the recombination-activating genes (Rag-1 or 2) prevent somatic rearrangement of the B and T cell receptors and thus impede development of these adaptive lymphocytes. In addition, deletion of the common gamma chain (γc) blocks signaling of IL-2, -4, -7, -9, -15, and -21, which abolishes the development of innate lymphoid precursors (IL-7 dependent) and IL-15-dependent NK cell differentiation. The only remaining immune compartment of mouse origin are murine myeloid cells, which can potentially phagocytose engrafted human cells ("Functional CD47/signal regulatory protein alpha (SIRP(alpha)) interaction is required for optimal human T- and natural killer- (NK) cell homeostasis in vivo.," 2011; "Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice.," 2011b).

The most commonly used NOD-scid $\gamma_c^{-/-}$ (NSG) or BALB/c RAG2$^{-/-}$ $\gamma_c^{-/-}$ (BRG) mouse backgrounds are then neonatally injected with CD34-positive hematopoietic progenitor cells (HPCs) (Ishikawa et al., 2005). After three months, nearly 60% of huMouse PBMCs tend to be of human origin (Rämer et al., 2011). Of the total human cells found in the periphery, B and T cells make up the bulk at around 45% each, with the majority of T cells being CD4$^+$, NK cells contribute another 5% and the rest of the compartment are monocytes (3%) and DCs (2%) (Rämer et al., 2011). Although these mice engraft most human immune system compartments, they lack gut mucosa reconstitution and germinal center development. The former can be resolved by implanting human fetal thymus and liver organoid under the kidney capsule, creating the so called bone marrow–liver–thymus (BLT) mice ("Cryptopatches are essential for the development of human GALT.," 2013). This for instance leads to T cells no longer being educated in the mouse thymus, but rather in the transplanted human organoid ("The analysis of the functions of human B and T cells in humanized NOD/shi-scid/gammac(null) (NOG) mice (hu-HSC NOG mice).," 2009). Taken together, mice such as the NSG, BRG or BLT mice are good models of the human immune system and are accordingly used to investigate human-tropic viruses, vaccines, etc.

PART VI – AIM AND OUTLINE

As outlined in the previous pages, NK cells are vital responders to a number of pathologies. Therefore, understanding their biology in more detail could help develop more specific therapies, which might take advantage of their natural cytotoxicity. Using humanized mice as a model organism, the goal was to analyze late human NK cell differentiation and repertoire development during steady state as well as under inflammatory conditions. Furthermore, in depth study of NK cell education could help decipher which of the models currently described for mouse NK cells best fits the actual *in vivo* process for human NK cells. Moreover, KIR dependent education can be uniquely studied in humanized mice, because this receptor family is not conserved in mice. Additionally, performing these phenotypic and functional studies of NK cells in huNSG, co-reconstituted with HLA-mismatched donors, allowed us to observe NK cell repertoire development and responses in conditions that mimic those of hematopoietic transplant patients. Apart from the study of NK cell development, we also wanted to explore the involvement of accessory cells in shaping NK cell responses. Therefore, in addition we investigated the cross-talk between pDCs and NK cells to understand the formation of this unique immunological synapse and how it could beneficially be targeted to improve NK cell responses.

CHAPTER THREE

NATURAL KILLER CELL REPERTOIRE DEVELOPMENT AND EDUCATION UNDER MHC CLASS I INFLUENCE IN TRANS

COGNATE HLA ABSENCE DIMINISHES HUMAN NK CELL EDUCATION IN TRANS

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3.1 ABSTRACT

Natural killer (NK) cells are innate lymphocytes with protective functions against virus infections and tumor formation. Human NK cells carry inhibitory killer cell immunoglobulin-like receptors (KIRs), recognizing distinct human leukocyte antigens (HLA). NK cells with KIRs for self-HLA molecules acquire superior cytotoxicity against HLA-negative tumor cells during education for improved missing-self recognition. Here we show that co-reconstitution of two KIR-ligand mismatched human immune system compartments in mice does not alter the frequency of KIR expressing NK cells, but their education. NK cell education is diminished for KIRs, whose cognate HLA molecules are lacking on leukocytes that reconstitute in parallel in the same mice. This change in NK cell education in mixed human donor reconstituted mice is functionally relevant, because it improves NK cell mediated immune control of Epstein Barr virus infection. Thus, leukocytes lacking cognate HLA ligands can disarm KIR-positive NK cells for improved immune control of a human γ-herpesvirus.

3.2 INTRODUCTION

Natural killer (NK) cells are the prototypic innate lymphocytes and have originally been identified by their ability to spontaneously kill transformed and infected cells (Herberman et al., 1975a; Kiessling et al., 1975; Trinchieri and Santoli, 1978). They recognize their targets by balancing signals of activating and inhibitory receptors, resulting in missing-self recognition upon loss of inhibitory ligands, mostly major histocompatibility complex (MHC) class I molecules, and altered-self recognition upon gain of activating ligands on the surface of encountered cells (Long et al., 2013; Martinet and Smyth, 2015; Moretta et al., 2001). The ability of NK cells to detect too few inhibitory ligands or too many activating ligands in reference to unaltered host tissue is thought to be acquired by NK cells in a continuous process called "education" or "licensing" via the interaction of inhibitory NK cell receptors and MHC class I molecules (Elliott and Yokoyama, 2011; Goodridge et al., 2015; Pradeu et al., 2013). Whether NK cell education is mediated "in cis" (on the NK cell itself) or "in trans" (by neighboring cells) or both is still a matter of debate (Bessoles et al., 2013; Chalifour et al., 2009; Ebihara et al., 2013; Joncker et al., 2010), however the hematopoietic compartment seems to be sufficient in both mouse and human for NK cell education (Ebihara et al., 2013; Haas et al., 2011). This process safeguards NK cell cytotoxicity by either "arming" cells that express in parallel to activating NK cell receptors inhibitory receptors that are sufficiently engaged by unaltered host cells, or by "disarming" through continuous engagement of activating receptors without inhibitory signals (Tripathy et al., 2008; Viant et al., 2014). While most activating NK cell receptors are nearly uniformly expressed on all mature NK cells, inhibitory receptors are expressed on subsets, whose contribution to the NK cell repertoire of an individual is guided by genetic and environmental factors, e.g. the genomic repertoire of killer immunoglobulin-like receptors (KIRs) and persistent cytomegalovirus (HCMV) infection in humans (Béziat et al., 2013). This composition of the NK cell repertoire becomes particularly important during primary infection with pathogens, like herpesviruses, which clinically identify individuals with primary immunodeficiencies that impair NK cell functions (Orange, 2013).

One of these is the infection with the human oncogenic γ-herpesvirus Epstein Barr virus (EBV). EBV persistently infects more than 90% of the human adult population (Taylor et al., 2015). While primary infection is usually asymptomatic in children, adolescents more frequently suffer from infectious mononucleosis (IM) during their first encounter with EBV (Balfour et al., 2013; Luzuriaga and Sullivan,

2010). While EBV is associated with B cell and epithelial malignancies through its viral oncogenes that are expressed during latent infection, lytic replication that produces infectious virions seems to be poorly controlled during IM and causes the characteristic immune pathogenic $CD8^+$ T cell lymphocytosis of this disease (Cesarman, 2014). NK cells were reported to expand during IM (Azzi et al., 2014; Balfour et al., 2013; Williams et al., 2005) and seem to particularly restrict lytic EBV infection that is deregulated during IM (Azzi et al., 2014; Chijioke et al., 2013). Interestingly and in contrast to the prototypic terminally differentiated NK cell expanding pathogen HCMV, EBV seems to drive proliferation of early differentiated non-KIR educated NK cells (Azzi et al., 2014; Dunmire et al., 2015). These NK cells have been reported to recognize lytically EBV replicating B cells via recognition by the activating NK cell receptors NKG2D and DNAM-1 (Pappworth et al., 2007), and the respective ligands for these receptors seem to be up-regulated during transition from latent to lytic EBV infection (Azzi et al., 2014; Pappworth et al., 2007). Loss of NK cells leads to elevated viral loads of wild-type, but not lytic replication incompetent EBV in vivo (Chijioke et al., 2013). This in turn causes hallmarks of IM, including enhanced $CD8^+$ T cell expansion as well as lymphadenopathy, and in addition weight loss and EBV associated tumorigenesis in EBV infected experimental animals (Chijioke et al., 2013). Moreover, the protective early differentiated NK cell subset decreases in frequency within the NK cell repertoire of humans during the first decade of life (Azzi et al., 2014), and therefore could explain the increased susceptibility to IM in adolescents. Thus, EBV infection seems to be sensitive to the balance between non-KIR educated NK cells, who are either KIR negative or whose KIRs have not encountered cognate MHC class I molecules, and KIR educated NK cells.

In our study we have investigated NK cell repertoire generation, NK cell education and its influence on the control of EBV infection in mice with reconstituted human immune system components (huNSG mice). For this purpose we have either reconstituted human NK cell compartments from hematopoietic progenitor cells that homozygously carry MHC class I molecules for distinct KIRs individually or in mixed chimeras. We found that the distribution of KIRs on the reconstituted human NK cells is donor autonomous and independent of environmental MHC class I molecules, while education, mediated by the hematopoietic compartment in this setting, is compromised as soon as a subset of immune cells does not carry the cognate MHC class I molecules. Thus, the abrogation of the educational process seems to be dependent on trans-interactions with co-reconstituting hematopoietic cells. This results in higher

frequencies of non-educated NK cells in the mixed reconstitutions and an improved immune control of EBV infection. Therefore, our findings are most consistent with a disarming model of KIR mediated education, resulting in NK cell compartments, which due to the inflammatory environment of EBV infection, nevertheless, efficiently control this human tumor virus.

3.3 RESULTS

3.3.1 A novel in vivo model with parallel reconstitution of two donors' immune compartments in NSG mice

Double-unit umbilical cord blood transplantation is successfully being used to improve engraftment and to decrease mortality in patients (Barker et al., 2005; Scaradavou et al., 2013). During the engraftment process, mostly one donor prevails which leads to single unit engraftment (Gutman et al., 2010; Hashem and Lazarus, 2015). Human NK cell education however has been shown to be driven by the hematopoietic compartment (Haas et al., 2011). Therefore, little data is available on how the absence of cognate human MHC class I molecules, namely cognate human leukocyte antigen (HLA) class I, on hematopoietic cells influences the education and KIR repertoire of human NK cells in trans. In order to study the effect of cognate versus non-cognate HLA on NK cell development, we reconstituted NOD-*scid* $\gamma_c^{-/-}$ (NSG) mice with HLA typed human hematopoietic progenitor cells (HPC) isolated from fetal livers (huNSG mice). Human fetal liver (HFL) donors were chosen to be homozygous for the HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4), which are recognized by KIR2DL and KIR3DL molecules, respectively. Furthermore, donors were disparate for HLA-A2 expression, which allowed us to track the progeny of specific donors in mixed reconstitution experiments (Figure 1A). Three types of experimental mouse groups were reconstituted in this study. Two single reconstituted groups homozygous for either HLA-C1 (recognized by KIR2DL2/3) or -C2 (recognized by KIR2DL1) and HLA-Bw4 (recognized by KIR3DL1) were set up in parallel to a third group that was reconstituted with a mix from both donors. Reconstitution of human $CD45^+$ leukocytes in general as well as frequencies of reconstituted T cells, B cells and NK cells were comparable to previous data generated in our lab (Figure 1B) (Strowig et al., 2010; 2009). Notably, mixed reconstituted huNSG mice developed cells from both donors side-by-side in all the reconstituted human immune system compartments (Figure 1C). Thus we established a model to study the influence of cognate HLA presence or absence in trans on NK cell education and KIR repertoire development.

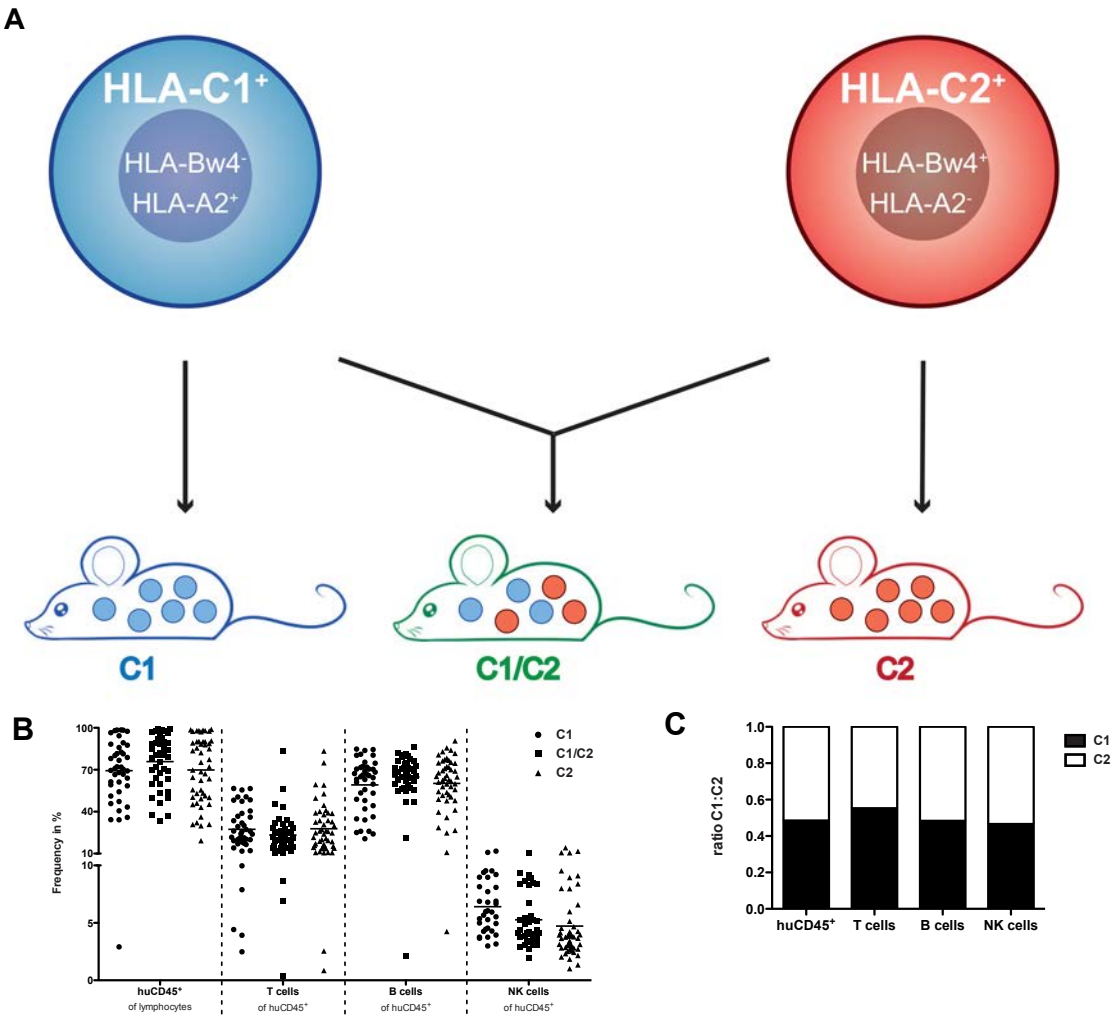

Figure 1. Mixed reconstitution of human immune system compartments from HLA mis-matched hematopoietic progenitor cells in NSG mice.

(A) Representative experimental overview. Three types of experimental groups, two reconstituted homozygously for HLA-C and -B allotype (HLA-C1, -C2 and -Bw4) while disparate for HLA-A2 and the third with a mix of both, were used. (B) Reconstitution of human immune cell compartments in the three experimental groups as percentage of human CD45+ lymphocytes. (C) Ratio of HLA-C1 donor vs. HLA-C2 donor frequencies as distinguished by HLA-A2 expression in immune cell compartments of mixed reconstituted huNSG mice. Data are pooled data from at least four independent experiments. n=34-49, bar represents mean in the respective graphs.

3.3.2 Development of the KIR repertoire on NK cells is not influenced by the HLA haplotype in trans

Next we analyzed the KIR repertoire of NK cells in liver and spleen of reconstituted huNSG mice and compared it to the KIR repertoire as present in the fetal liver of the original donor (Figure 2, Figure S1 for gating, Figure S2 and data not shown). In order to detect differences in mixed reconstituted huNSG mice, NK cells from these mice were separately evaluated as per donor origin. The overall diversity of the KIR repertoire was comparable among all groups as well as to the donor HFLs and no preferential expansion of KIR subsets could be seen (Figure 2). When relating the KIR frequencies of reconstituted mice to their specific HFL donors, a correlation could be detected between the two (Figure S2). Namely, HFL donors with for example high frequencies of KIR2DL1, KIR2DL2/3 or KIR3DL1 single positive NK cells reconstituted the respective NK cell subsets also at higher frequencies. Importantly, in mixed reconstituted huNSG mice, the presence of non-cognate HLA in trans did not significantly change the KIR repertoire (Figures 2B and 2C) and no differences were detectable when comparing specific KIR frequencies to those of single reconstituted mice (Figure S2). In spleen, a similar KIR repertoire composition as in the liver was observed, but could not be compared with the splenic NK cell repertoire of the HFL donors (data not shown). Hence it seems that the absence of cognate HLA on co-reconstituting hematopoietic cells does not influence the development of the KIR repertoire in the steady state.

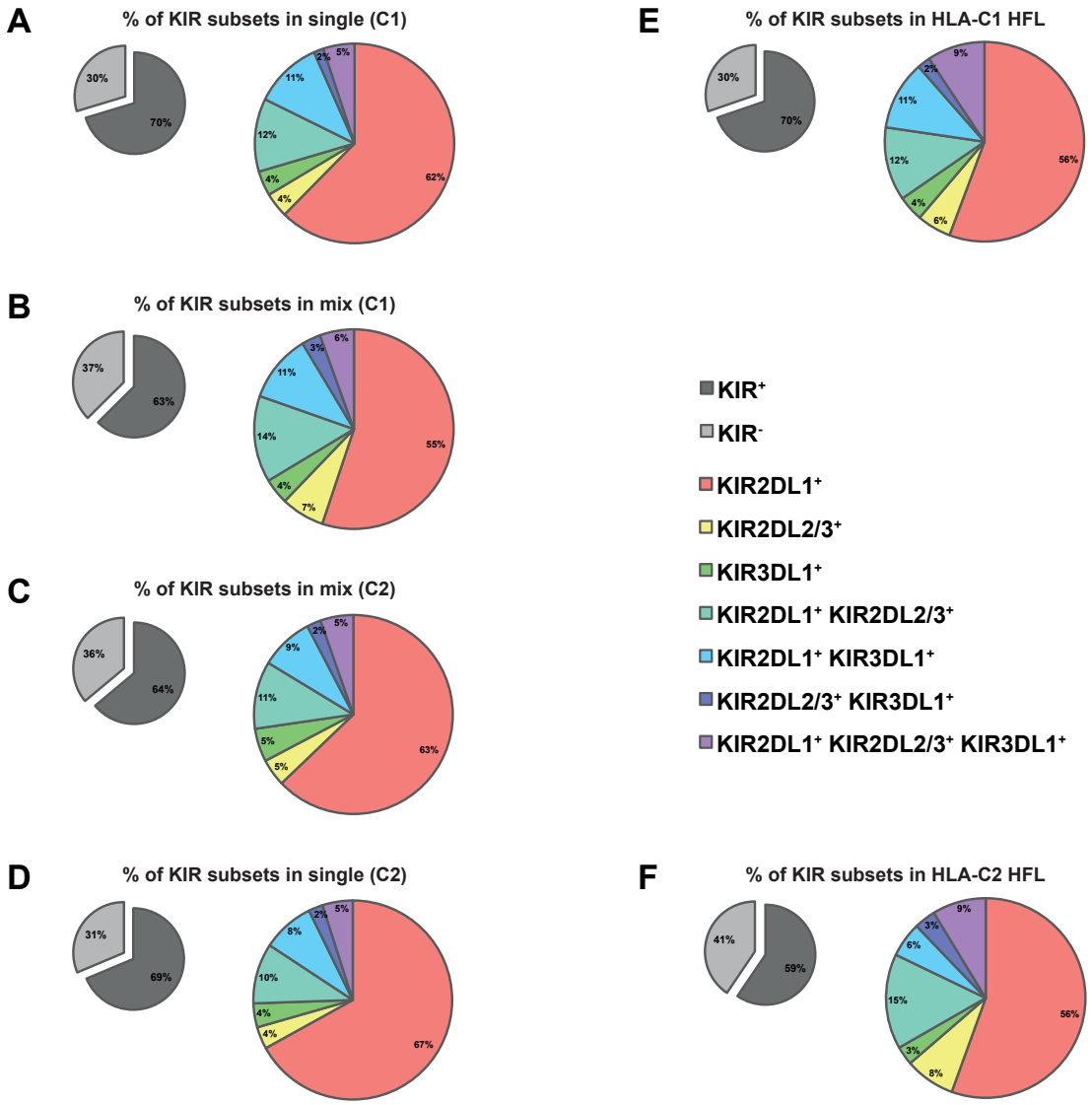

Figure 2. HLA haplotype in trans does not influence KIR repertoire in steady state

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix). Grey pie charts depict the frequencies of KIR^+ and KIR^- NK cells and colored pie charts represent the indicated KIR^+ NK cell subsets.

(A to F) Representation of KIR expressing NK cell subsets as percentages of total KIR^+ NK cells in liver of huNSG mice (A to D) or human fetal liver (HFL) donors (E and F). KIR subsets of liver NK cells from huNSG mice reconstituted with single donors homozygous for HLA-C1 (A) or -C2 (D). (B and C) KIR subsets of liver NK cells from mixed donor reconstituted huNSG mice, with NK cells derived from HLA-C1 donor (B) and -C2 donor (C). (E and F) KIR subsets of HFL NK cells from HLA-C1 (E) or -C2 HFLs (F). n=3. Data in (A) to (D) are pooled data from at least three independent experiments. n =11-14 mice per group.

3.3.3 EBV infection does not force skewing of the KIR repertoire on NK cells in huNSG mice

Previous work has established profound effects of human CMV infection on the NK cell KIR repertoire and expansion of KIR educated NKG2C$^+$ NK cells (Béziat et al., 2013; Gumá et al., 2004). In an effort to examine if the KIR repertoire could be skewed by another herpesvirus, huNSG mice were infected with Epstein Barr virus (EBV) and monitored for 5 weeks. At endpoint, livers and spleens of infected animals were analyzed for KIR expression in NK cells (Figure 3, Figure S3). EBV infection did not lead to obvious changes in the KIR$^+$ NK cell subset composition in single and mixed reconstituted huNSG mice (Figures 3A-B and Figures 3C-D). Furthermore, when comparing frequencies of NK cells single positive for KIR2DL1, KIR2DL2/3 or KIR3DL1 in EBV infected animals to controls, no differences could be detected (Figures 3E-P). Stable KIR repertoires were also observed in livers of EBV infected huNSG mice (Figure S3). It therefore appears that EBV infection is not able to induce skewing of the KIR repertoire in huNSG mice.

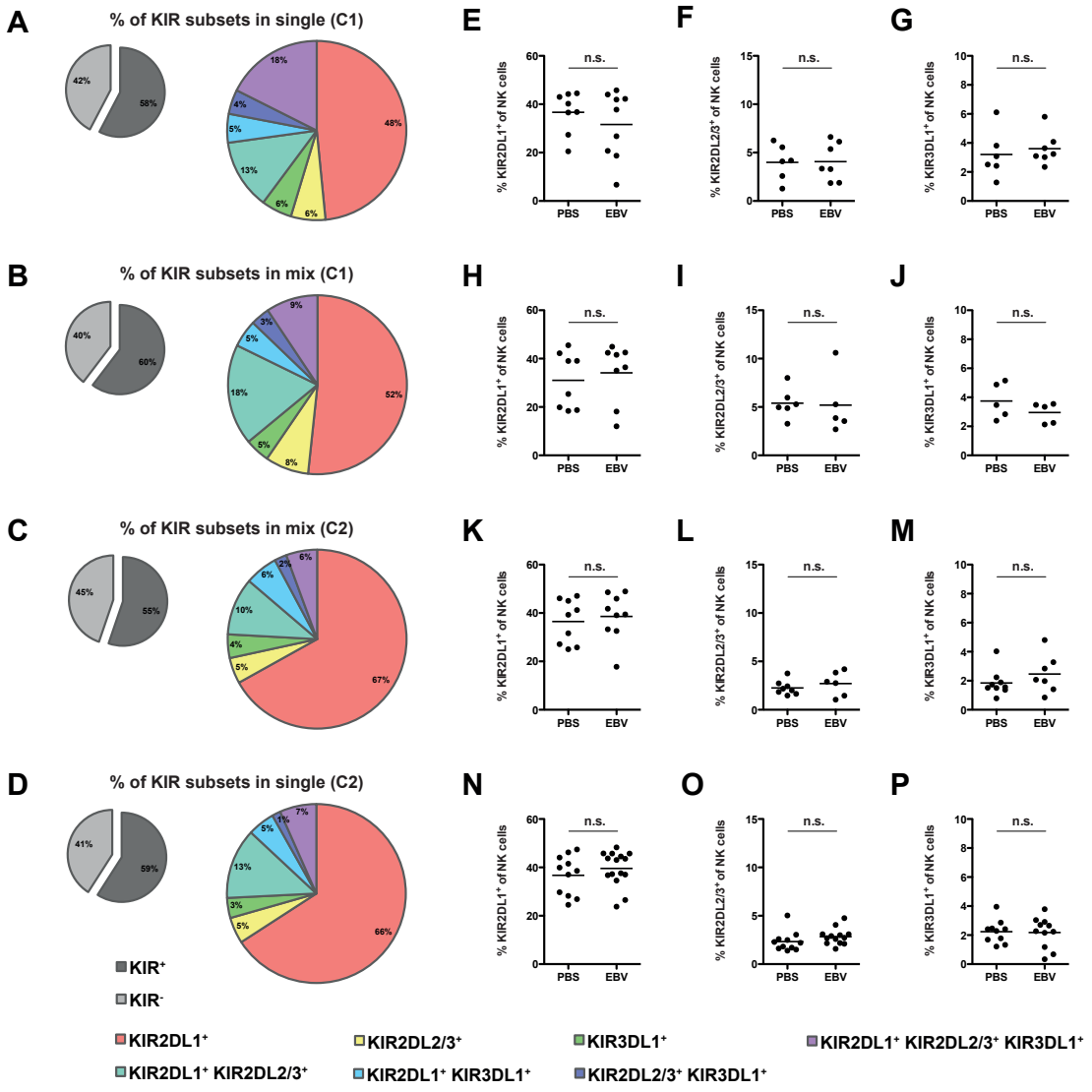

Figure 3. Infection with EBV does not lead to skewing of KIR repertoire

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix). Grey pie charts depict the frequencies of KIR$^+$ and KIR$^-$ NK cells and colored pie charts represent the indicated KIR$^+$ NK cell subsets.

(A to D) Subsets of KIR-expressing NK cells as percentages of total KIR$^+$ NK cells in spleen of EBV infected huNSG mice. (E to P) Frequency of single KIR$^+$ NK cells in spleens of huNSG mice at endpoint. Comparison of PBS controls to EBV infected animals for frequency of KIR2DL1$^+$, KIR2DL2/3$^+$ or KIR3DL1$^+$ NK cells in homozygous reconstituted mice with HLA-C1 donor (E to G), mixed reconstituted mice with cells derived from HLA-C1 donor (H to J) or -C2 donor (K to M) and homozygous reconstituted mice with HLA-C2 donor (M to P).

Data are pooled data from at least three independent experiments. n=8-15 mice per group, bar represents mean, unpaired t test, n.s., not significant.

3.3.4 Absence of cognate HLA abrogates NK cell education in huNSG mice

Whether human NK cells in mouse models reconstituted with human immune system components are educated has not been addressed so far. To quantify education in huNSG mice, we assessed degranulation of single KIR^+ NK cells towards a prototypic NK cell target, the MHC-class I negative erythroleukemic K562 cells, in the context of the educating HLA ligands. The presence of solely the educating, cognate HLA as in the case of single reconstituted huNSG mice resulted in education of KIR2DL1, KIR2DL2/3 and KIR3DL1 positive NK cells in HLA-C2, - C1 and -Bw4 reconstituted animals respectively (Figures 4A-C), with functional differences similar to the differences between educated and non-educated NK cells from healthy volunteers (Charoudeh et al., 2012; 2006). Furthermore, acute infection with EBV did not influence this process. In contrast however, the additional presence of non-cognate HLA in trans in mixed reconstituted huNSG mice prevented NK cells from being educated (Figures 4A-C). Interestingly, education was lost regardless of the donor ratio in mixed reconstituted animals and even observed when the cognate HLA negative leukocyte reconstitution only amounted to 5% of the total human immune components (Figure S4). This argues against a dose effect of cognate HLA and for the disarming model in KIR mediated NK cell education. When looking at education in the context of NKG2A and its ubiquitously present educating HLA-E ligand, both mixed and single reconstituted huNSG mice displayed equal education, consistent with invariant HLA-E expression by all HFL donors (Figure 4D). Therefore, education of human NK cells takes place in huNSG mice, independently of EBV infection, is mediated by the hematopoietic compartment and is reliant on the presence of cognate HLA on all hematopoietic cells. Furthermore, the abrogation of NK cell education in mixed reconstituted animals is dependent on trans- interactions.

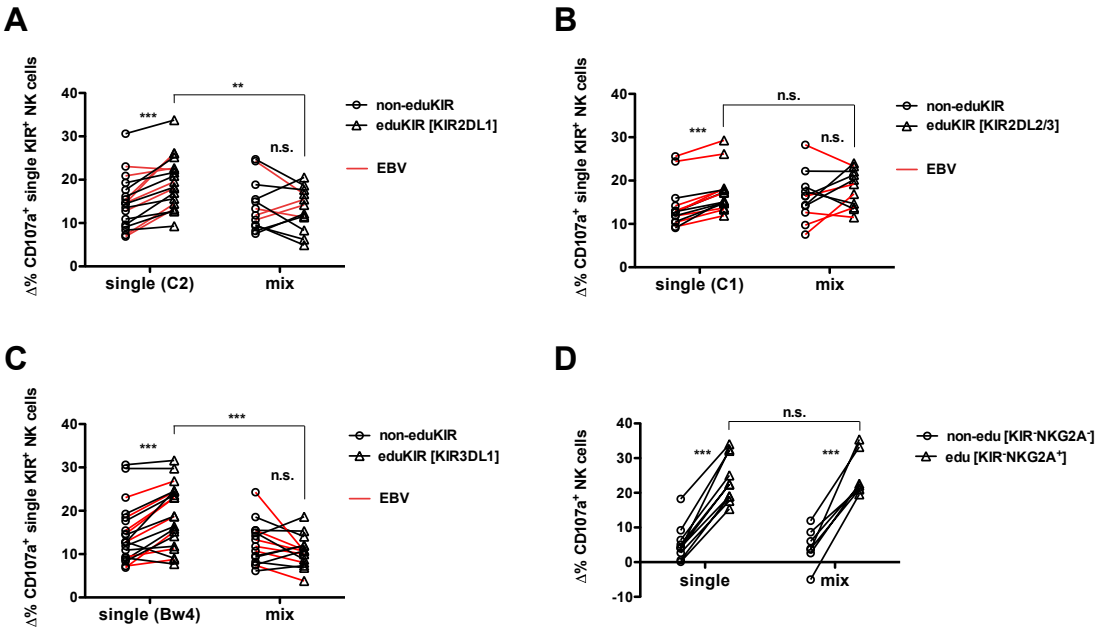

Figure 4. Loss of NK cell education in the presence of non-cognate MHC class I in mixed reconstituted huNSG mice.

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix).

(A to C) Degranulation of single KIR-positive NK cells after incubation of K562 cells with splenocytes from huNSG mice reconstituted with (A) homozygous HLA-C2 or mixed (n=12-18 mice per group), (B) homozygous HLA-C1 or mixed (n=11-14 mice per group) or (C) homozygous HLA-Bw4 or mixed donors (n=15-20 mice per group), infected (red lines) or not (black lines) with EBV for five weeks. According to single reconstituted allotypes, eduKIR denotes educated NK cells and non-eduKIR denotes non-educated NK cells gated on cells derived from donors used for single reconstitution in both single and mixed reconstituted huNSG mice, respectively.

(D) Degranulation of KIR$^-$ NKG2A$^-$ (non-edu) and KIR$^-$ NKG2A$^+$ (edu) NK cells after incubation of K562 cells with splenocytes from single or double (mix) reconstituted huNSG mice (n=7-10 per group), gated on cells derived from donors used for single reconstitution.

Data in (A) to (C) are pooled data from at least three independent experiments; data in (D) are compound data from two independent experiments. Data were analyzed by multiple t tests. n.s., not significant, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3.5 Non-educated NK cells still display inhibition in response to cognate HLA

In addition to analyzing education of human NK cells in huNSG mice, we also studied the inherent inhibitory potential of specific KIR receptors. To do so, we tested degranulation towards K562 cells stably expressing individual HLA-C1, -C2 or -Bw4 molecules. Both educated and non-educated NK cells that carry a single KIR specific to the transfected HLA on the target cell showed reduced levels of degranulation compared to unaltered or non-cognate HLA transfected K562 cells (Figure 5; data not shown). Thus, despite the loss of education in NK cells from mixed reconstituted mice, non-educated single KIR^+ NK cells still dampened their response and exhibited self-inhibition, as previously observed for NK cells of healthy volunteers (Yu et al., 2007). In addition to a reduced education, the inhibition of NK cell reactivity by KIR-ligand expressing targets likely contributes to NK cell tolerance in mixed chimeras.

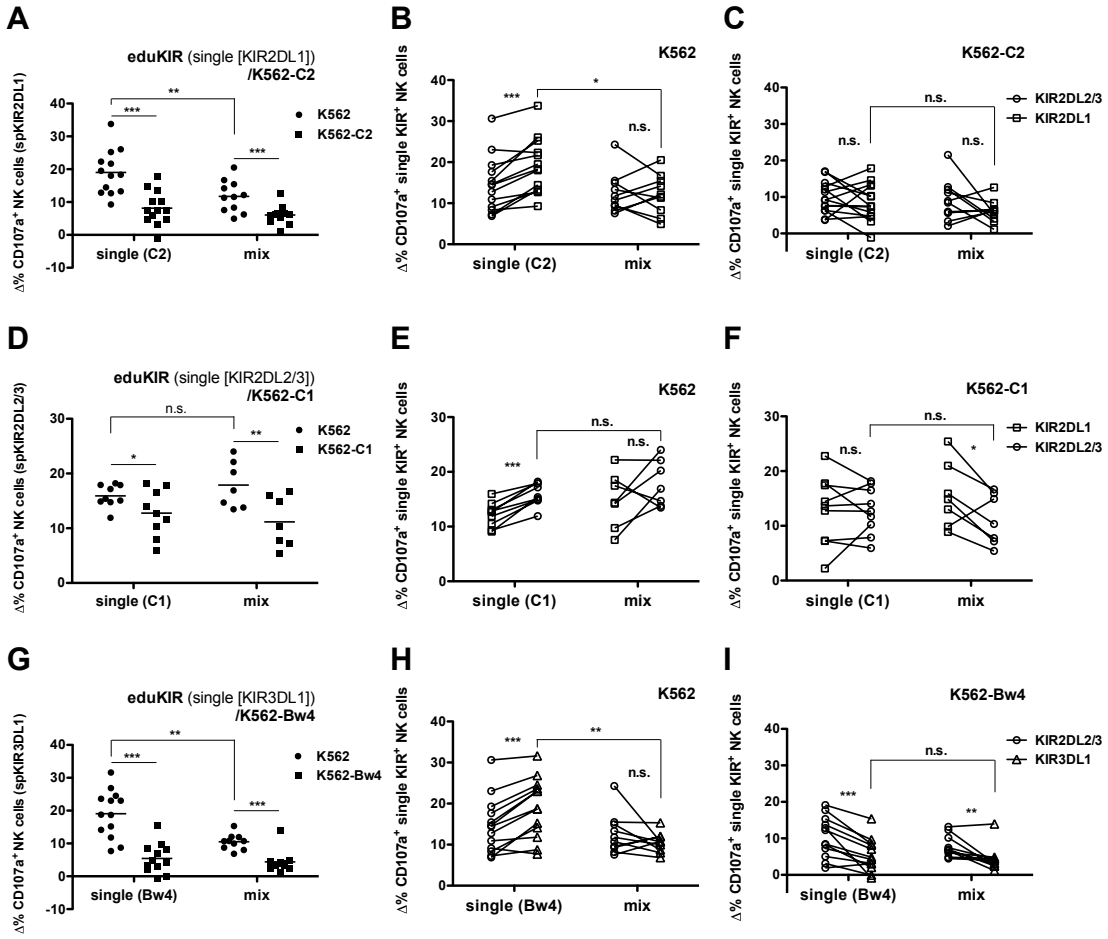

Figure 5. Inhibition by cognate MHC class I ligands is maintained in non-educated NK cells.

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix) and incubated with MHC class I negative target cells (K562) or K562 transfectants expressing HLA-C1, -C2 or -Bw4 MHC class I ligands. According to single reconstituted allotypes, eduKIR denotes educated NK cell subset gated on cells derived from donor used for single reconstitution in both single and mixed reconstituted huNSG mice.

(A to C) Degranulation of single KIR2DL1$^+$ NK cells after incubation of K562 or K562-C2 cells with splenocytes from huNSG mice reconstituted with HLA-C2 homozygous or mixed donors (A) and paired analysis for incubation with K562 cells (B) or K562-C2 transfectants (C).

(D to F) Degranulation of single KIR2DL2/3$^+$ NK cells after incubation of K562 or K562-C1 cells with splenocytes from huNSG mice reconstituted with HLA-C1 homozygous or mixed donors (D) and paired analysis for incubation with K562 cells (E) or K562-C1 transfectants (F).

(G to I) Degranulation of single KIR3DL1$^+$ NK cells after incubation of K562 or K562-Bw4 cells with splenocytes from huNSG mice reconstituted with HLA-Bw4 homozygous or mixed donors (G) and paired analysis for incubation with K562 cells (H) or K562-Bw4 transfectants (I).

Data are pooled data from at least two independent experiments and were analyzed by multiple t tests. Bar represents mean on relevant graphs. n=7-14 mice per group, n.s., not significant, *p <0.05, **p < 0.01, ***p < 0.001.

3.3.6 Mixed reconstituted huNSG mice display superior immune control towards EBV

In order to assess the functional consequence of reduced NK cell education, we evaluated immune control of EBV infection. It had been previously shown that non-educated NK cells are mainly responsible for the innate immune response to mouse cytomegalovirus (MCMV) infection (Orr et al., 2010). Therefore, we investigated if an increased abundance of non-educated NK cells would improve immune control of EBV infection. To follow disease progression of EBV infected huNSG mice, viral loads were assessed on a weekly basis in blood and in spleen at the experimental endpoints. Viral titers were significantly reduced in both blood and spleen of mixed reconstituted animals at the endpoints of the experiments, when compared to EBV infection of single donor reconstituted animals (Figures 6A-D). Furthermore, we also observed a reduced $CD8^+$ T cell expansion in response to EBV infection in mixed reconstituted huNSG mice (Figure 6E), which indicates a lower viral burden and antigen amount. However, the decreased conversion to $CD8^+$ T cells in mixed reconstituted animals did not result in an altered $CD8^+$ T cell composition (Figures S5A-H), nor were there differences in the $CD8^+$ T cell effector memory response of the various groups (Figure S5I). Thus, side-by-side reconstitution of huNSG mice with two donors, which leads to less educated NK cells, allows for improved immune control against EBV infection.

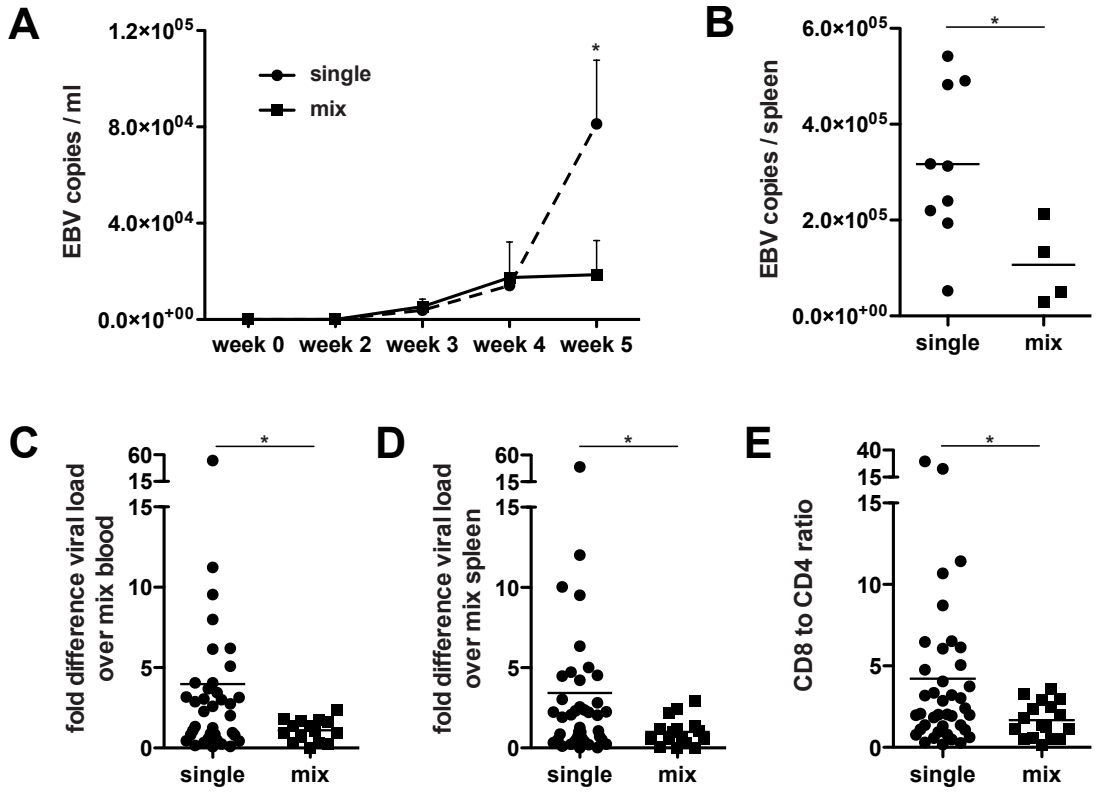

Figure 6. Improved EBV specific immune control in mixed reconstituted huNSG mice

Viral loads of huNSG mice were read out over time in blood and at endpoint in spleen. Homozygous single reconstituted huNSG (single) were compared to mixed reconstituted animals (mix).

(A and B) Viral loads in blood at weekly time points (A) and in spleen (B) of single vs. mixed reconstituted huNSG mice for a representative experiment (n=3-9 mice per group, A, mean ± SEM, ANOVA with Bonferroni correction).

(C and D) Fold difference in viral load for blood (C) and spleen (D) at endpoint normalized to mean of mixed reconstituted group.

(E) $CD8^+$ to $CD4^+$ T cell ratio in blood at endpoint in single vs. mixed reconstituted huNSG mice.

Data in (C) to (E) are pooled data from at least three independent experiments. n=15-40, bar represents mean, Mann-Whitney or unpaired t test where appropriate, *$p < 0.05$.

3.3.7 NK cells mediate improved immune control of EBV infection in mixed reconstituted mice

To elucidate a possible role of NK cells in the improved immune control of EBV, huNSG mice were depleted of NK cells prior to infection with EBV. Interestingly, depletion of NK cells leveled the differences previously observed in viral loads between mixed reconstituted and single reconstituted huNSG mice (Figure 7A). Moreover, viral titers in both blood and spleen of mixed reconstituted animals depleted of NK cells were significantly higher compared to viral loads in mixed reconstituted animals without depletion of NK cells (Figures 7B and 7C). Additionally, the loss of NK cell mediated protection correlated with an elevated $CD8^+$ T cell expansion in NK cell-depleted mixed reconstituted huNSG mice (Figure 7D). Finally, in order to investigate NK cell memory formation upon EBV infection, we assessed surface markers that had been implicated in NK cell memory formation during viral infections (Lee et al., 2015; Schlums et al., 2015). Phenotypically, however, we could not detect differences between single and mixed reconstituted huNSG mice in educated or non-educated NK cells, exemplified by the expression of CD2, as a possible marker of memory NK cells (Figure S6). These data indicate that non-educated NK cells might play a key role in the improved control of EBV infection in mixed reconstituted huNSG mice, but do not form a phenotypically identifiable memory NK cell compartment.

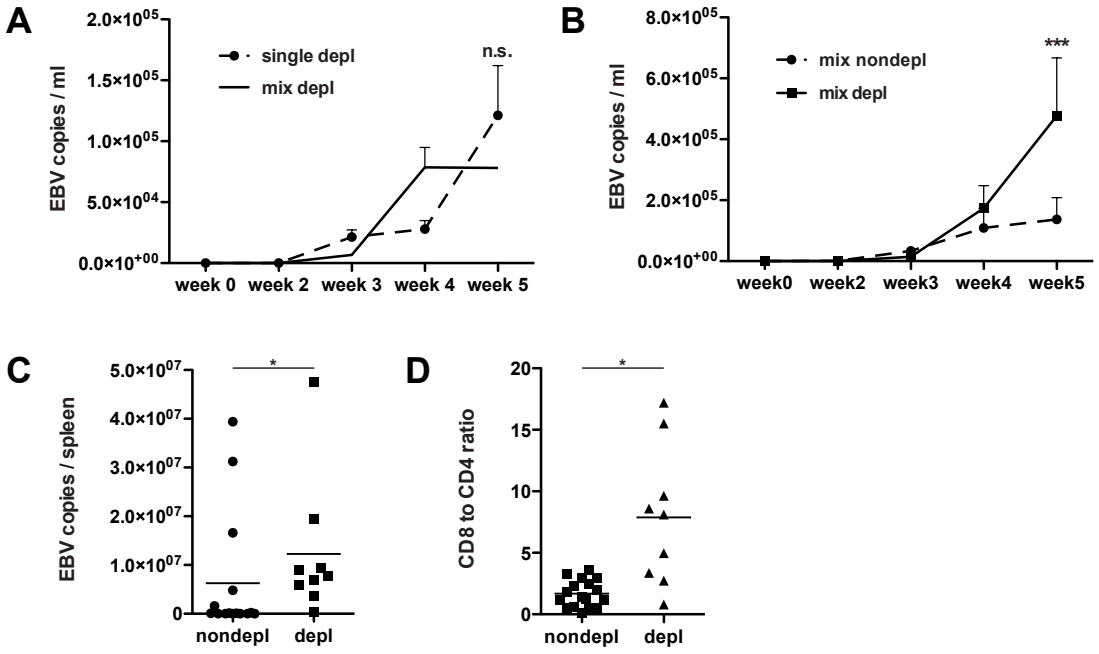

Figure 7. NK cells mediate protection in mixed reconstituted huNSG mice

Viral loads of huNSG mice were read out over time in blood and at endpoint in spleen. Homozygous single reconstituted huNSG were compared to mixed reconstituted animals and non-NK cell-depleted (nondepl) were compared to NK cell-depleted (depl) mixed reconstituted huNSG mice.

(A) Representative experiment for viral loads in blood at weekly time points of NK cell-depleted single vs. NK cell-depleted mixed reconstituted huNSG mice (n=3-8 mice per group).

(B) Viral loads in blood over time of non-depleted (nondepl) vs. NK cell-depleted (depl) mixed reconstituted huNSG mice.

(C) Viral loads in spleen at endpoint of non-depleted (nondepl) vs. NK cell-depleted (depl) mixed reconstituted huNSG mice.

(D) $CD8^+$ to $CD4^+$ T cell ratio in blood at endpoint of non-depleted (nondepl) vs. NK cell-depleted (depl) mixed huNSG mice.

Data in (B) to (D) are pooled data from at least two independent experiments. n=8-15 mice per group, mean ± SEM or bar represents mean. Data were analyzed by ANOVA with Bonferroni correction, Mann-Whitney or unpaired t test where appropriate, n.s., not significant, *$p < 0.05$, ***$p < 0.001$.

3.4 DISCUSSION

Using mixed hematopoietic progenitor cell reconstitution in NSG mice, we were able to assess the influence of cognate HLA absence in trans on KIR repertoire generation and NK cell education in the reconstituting human immune system. We observed that similar repertoires of KIR positive NK cells reconstituted in single donor and mixed KIR-ligand mismatched donor reconstituted NSG mice as well as in the fetal liver of the HPC donors used for huNSG generation. In contrast, KIR mediated NK cell education was compromised if even a small subset of leukocytes that lacked the respective KIR-ligand reconstituted in the same NSG mouse in parallel. This indicates that KIR mediated education seems to be even more sensitive than Ly49 mediated education, with disarming taking place at a KIR-ligand negative frequency below 10% compared to 20% for the required Ly49 ligand negative frequency in the murine system (Johansson and Höglund, 2004; Johansson et al., 1997). Therefore, our findings suggest that such small KIR-ligand negative leukocyte populations are able to disarm NK cells for decreased missing-self recognition. However, our data suggest that the availability of a low affinity binding ligand, as is the case for the KIR2DL2/3-ligand HLA-C2 (Pende et al., 2009), can be sufficient to prevent disarming of NK cells in trans (Figures 4B, 5D and E). Hence the mechanism of disarming and which leukocyte populations are mainly involved require further investigations. Nevertheless, disarming, while disabling HLA negative tumor cell recognition, improves NK cell mediated immune control of herpesvirus infections, as previously shown for the γ-herpesvirus MCMV (Orr et al., 2010) and shown here for the γ-herpesvirus EBV (Figures 6 and 7).

This study addresses for the first time KIR mediated NK cell education by cognate and non- cognate HLA molecules on neighboring leukocytes, because this family of inhibitory NK cell receptors is absent in mice (Long et al., 2013) and during transplantation with double cord blood units usually one donor eliminates the other due to alloreactive responses by co-transferred T cells (Gutman et al., 2010). Therefore, KIR mediated NK cell education cannot be studied in these settings. Nevertheless, understanding KIR mediated NK cell education is of clinical importance, because it has been shown that haploidentical hematopoietic cell transplantation, harnessing KIR-HLA mismatch recognition, provides therapeutic benefits in adult acute myeloid leukemia (AML) and pediatric acute lymphoblastic leukemia (ALL) patients (Locatelli et al., 2015; Ruggeri et al., 2002; Velardi et al., 2012) (46). It is thought that NK cells reconstituting from the KIR-ligand mismatched graft mediate a graft-versus-leukemia

(GvL) effect via missing-self recognition of the leukemic blasts. Indeed, it has been observed that the reconstituting NK cells mediate allorecognition against the recipient's leukemic blasts to a similar extent as the donor's mature NK cells, and that the degree of donor allorecognition of recipient cells is the best predictor for clinical efficacy (Pende et al., 2009; Pietra et al., 2015). These findings suggest that the donor's reconstituting human immune system components are sufficient to educate NK cells for KIR- ligand mismatched leukemia recognition, that the allogeneic residual leukemia blasts of the recipient are not able to sufficiently disarm the reconstituting NK cells and that also the recipient's somatic cells are not able to sufficiently induce tolerance in the reconstituting donor NK cells. However, our data suggest that this effect cannot be further potentiated by combining donors with high anti-leukemic NK cell reactivity, because their reconstituting leukocytes might disarm each other and result in diminished GvL reactivity, as observed in our study in the recognition of the human erythroleukemia cell line K562.

In contrast to possibly diminished GvL activity in mixed reconstitutions, the resulting mixed NK cell compartments might be superior in preventing EBV associated lymphomas during transplantation. Indeed, most post-transplant lymphoproliferative diseases (PTLDs) are EBV positive (Cesarman, 2014) and plasma EBV DNA copy number seems to be a reliable indicator of PTLD occurrence and treatment response (Ruf et al., 2012; Tsai et al., 2008). This might in part reflect uncontrolled lytic EBV replication in transplant patients prior to PTLD, infecting and transforming reconstituting B cells to result in poly- or oligoclonal lymphoproliferations. This lytic replication is indeed susceptible to NK cell mediated immune control and non-KIR educated NK cells have been shown to preferentially target lytically EBV replicating B cells (Azzi et al., 2014; Chijioke et al., 2013). Furthermore, in the presence of side-by-side KIR-ligand mismatched donor reconstitution, mismatched EBV-transformed B cells might be more susceptible to NK cell lysis, while the response to their matched counterpart would still be dampened by KIR inhibition (Yu et al., 2007). Therefore, mixed hematopoietic progenitor cell reconstitutions could be beneficial for controlling herpesvirus infections, resulting in mixed, less educated NK cell compartments that more efficiently control EBV infection, as shown in this study, and possibly also cytomegalovirus infection in humans.

Our study, therefore, contributes to an increasing body of literature that suggests that hematopoietic cell transplantation should be tailored to address

different therapeutic needs of patients. Completely HLA matched transplantation reduces the risk for graft-versus-host disease, but haploidentical HLA mismatched transplantation of T cell depleted grafts mediates a potent GvL effect by NK cell alloreactivity. In addition, mixed transplantation of T cell depleted grafts might alter NK cell education for more efficient herpesvirus specific immune control. In support of this notion, our data suggest that even minor leukocyte populations lacking cognate HLA molecules for KIRs might reduce education of the respective KIR carrying NK cell populations for improved EBV specific immune control.

3.5 METHODS

Mice

NOD/LtSz-scid IL2Rγ^{null} (NOD-scid $\gamma_c^{-/-}$ or NSG) mice were obtained from the Jackson Laboratory and bred and maintained at the Institute of Experimental Immunology, University of Zürich, Switzerland, under specific pathogen-free conditions. Newborn NSG mice (1 to 5 days old) were irradiated with 1 Gy using a Cs or X-ray source. Five to six hours after irradiation, mice were injected intrahepatically with 1–2 × 10^5 CD34$^+$ human hematopoietic progenitor cells (HPCs) derived from human fetal liver tissue obtained from Advanced Bioscience Resources. Injected HPCs were either from a single donor or a mix of two donors at a 1:1 ratio, amounting to the same total cell number. Preparation of human fetal liver tissue and isolation of human CD34$^+$ cells was done as described previously (Strowig et al., 2009; 2010; White et al., 2012). Reconstitution of human immune system components in mice (huNSG) was analyzed 10–12 weeks after engraftment and again just before the start of the experiments. Mice with more than 40% reconstitution of huCD45$^+$ human immune system components in the lymphocytes of peripheral blood were used in the described experiments (average 70% huCD45$^+$ cells of peripheral blood lymphocytes, 25% CD3$^+$ T cells of human lymphocytes, 60% CD19$^+$ B cells of human lymphocytes, 5% NK cells of human lymphocytes, 80% CD4$^+$, and 20% CD8$^+$ T cells of human T cells), were sex matched, and were 12–18 weeks old at the start of the experiments. All animal protocols were approved by the cantonal veterinary office of the canton of Zürich, Switzerland (protocol nos. 148/2011 and 209/2014). All studies involving human samples were reviewed and approved by the cantonal ethical committee of Zürich, Switzerland (protocol no. KEK-StV-Nr.19/08).

HLA typing

To perform HLA genomic typing, DNA was extracted using the Qiagen GmbH reagent, according to manufacturer's instruction. HLA alleles were identified by PCR–Sequence-Specific Oligonucleotide Reverse assay using commercial HLA Kits (Fujirebio Italia s.r.l.). Data analysis was performed using the LIPA Interpretation Software (Fujirebio).

Virus Infection and NK Cell Depletion

GFP-Epstein-Barr virus (EBV) B95-8 wild-type was produced in 293 cells. Titration of viral concentrates was done on Raji cells in serial dilutions and calculated as Raji-infecting units (RIU) using flow cytometric analysis of GFP-positive Raji cells 2 days after infection of cells. HuNSG mice were infected via intraperitoneal injection of 1 × 105 RIUs and followed for 5 weeks. Depletion of NK cells in huNSG mice was done via intraperitoneal injection of purified anti-NKp46 antibody (clone BAB281) in PBS on three consecutive days (total of 300 μg per mouse). On the following day, animals were infected by inoculation via intraperitoneal injection of 1 × 105 RIU EBV.

Flow Cytometry

All fluorescently labeled antibodies were purchased from BD Biosciences, Beckman&Coulter, BioLegend, Invitrogen and R&D Systems (Table S1). Spleens were mechanically disrupted and filtered through a 70 μm cell strainer before separation of mononuclear cells on Ficoll-Paque gradients. Livers were first digested in a buffer containing DNase and CollagenaseD (Roche). After being mechanically disrupted, mononuclear cells were separated using a Percoll gradient. Lysis of erythrocytes in whole blood was done with NH_4Cl. For intracellular staining, the Cytofix/Cytoperm Kit from BD Biosciences was used. Cell suspensions were stained with antibodies for 30 min on ice, washed, and analyzed on FACSCanto or LSR Fortessa cytometers (BD Biosciences). Analysis of flow cytometric data was performed with FlowJo (Tree Star).

K562 cells and their HLA class I transfection

K562 cells were maintained in RPMI 1640 medium (GIBCO BRL) supplemented with gentamicin (20μg/ml) and 10% FCS (GIBCO BRL). Cell cultures were kept in a water-saturated atmosphere with 5% CO_2 at 37°C. For the generation of HLA-Bw4, HLA-C1 or HLA-C2-expressing K562 cells, K562 cells were transfected with HLA-B*51:01, HLA-C*03:03 or HLA-C*05:02 cDNA, respectively, inserted into the expression vector pcDNA3.1 (Invitrogen, Groningen, Netherlands). Briefly, K562 cells were transfected by electroporation with plasmid DNA (10μg/1x10^6 cells) in OPTIMEM1 with Glutamax (GIBCO BRL). Electroporation conditions were 250V, 300μF using the Gene Pulser ® II (BioRad). Transfected cells were selected by the addition of G418 (1 mg/ml) to the culture medium and subsequently cloned by single cell sorting of HLA-positive cells using flow cytometry.

Degranulation Assay

For the degranulation assay, splenic cell suspensions and non-transfected K562 or HLA-transfected K562 cells were cocultured at a ratio of 10:1 for 5 h with addition of monensin after 1 h, and NK cells were then analyzed for expression of the degranulation marker CD107a. Single KIR-positive NK cells were not pre-gated on NKG2A.

Quantification of EBV load by real-time PCR

Total DNA from the whole blood was extracted using NucliSENS EasyMAG system (bioMérieux) according to manufacturer's instructions. Splenic tissue was processed using using QIAamp DNA tissue Kit (QIAGEN) using the manufacturer's protocol. Quantitative analysis of EBV DNA was performed by TaqMan (Applied Biosystems) real-time PCR technique as described (51) with modified primers for the BamH1 W fragment (5′-CTTCTCAGTCCAGCGCGTTT-3′ and 5′- CAGTGGTCCCCCTCCCTAGA-3′) and a fluorogenic probe (5′-(FAM)- CGTAAGCCAGACAGCAGCCAATTGTCAG-(TAMRA)-3′). All PCRs were run on an ABI Prism 7700 Sequence Detector (Applied Biosystems) and samples were analyzed in duplicates. No EBV DNA was detected in the blood of mock-infected animals for the duration of the experiment. Mice were considered uninfected if EBV DNA was not detected in the blood and spleen during the experiment.

Statistics

All data were analyzed with an unpaired two-tailed Student's t test, Mann-Whitney t test or ANOVA with Bonferoni correction as stated. A p value of < 0.05 was considered statistically significant. Statistical analysis and generation of graphs was performed with Prism software (GraphPad).

3.6 AUTHOR CONTRIBUTIONS

V.L. and A.R. conducted the experiments. G.P. and G.F. performed the HLA typing. V.B. and K.J.M. were instrumental for the KIR repertoire analysis. E.M. and A.M. provided essential reagents for the study. C.G. generated the K562 transfectants. R.C. and A.Z. determined the viral titers. V.L., O.C. and C.M. designed the experiments, analyzed the data and wrote the manuscript.

3.7 ACKNOWLEDGMENTS

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3.8 SUPPLEMENTAL FIGURES

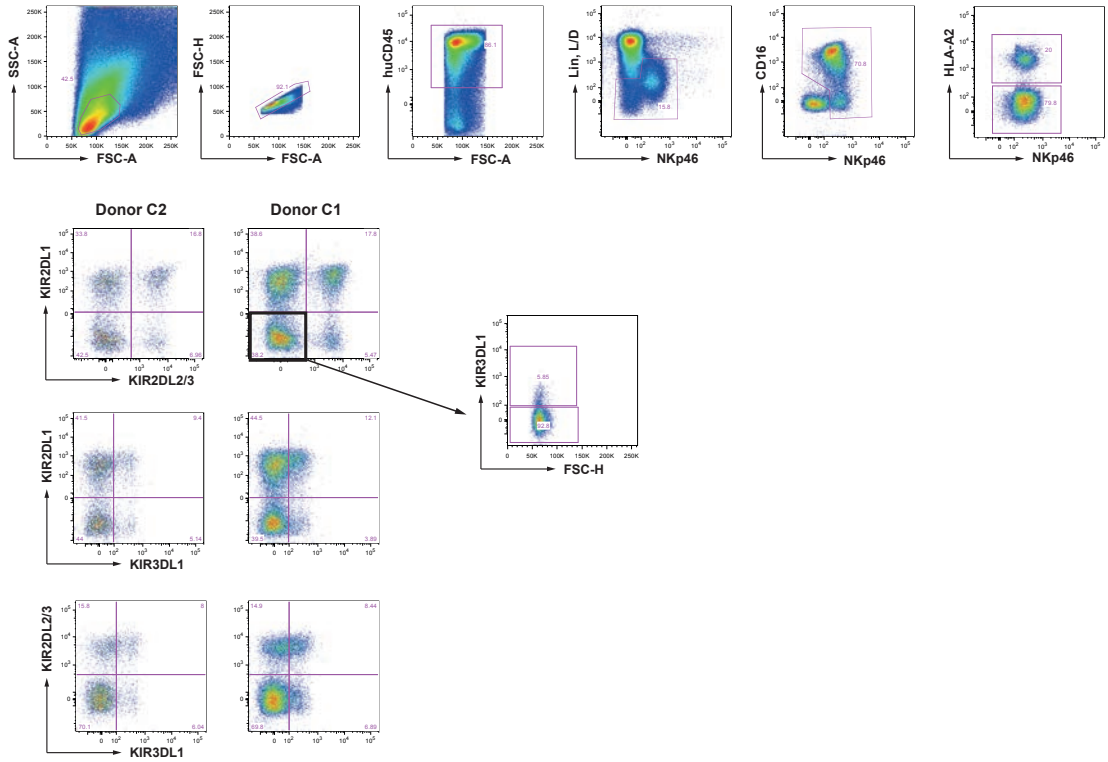

Figure S1, related to Figure 2. Gating strategy of KIR subsets.

Spleen and livers of huNSG were processed and stained with fluorescent markers for flow cytometry. Cells were pre-gated on lymphocytes and singlets before discriminating for the huCD45$^+$ population. HuCD45$^+$ lymphocytes were gated on Lin$^-$, L/D$^-$ vs. NKp46. NKp46$^+$ cells were further characterized by CD16 to arrive at the NK cell population. NK cells were assessed according to HLA-A2 for their donor origin and then gated on KIR expression. To arrive at single positive KIR populations, double negative quadrants were gated on the third parameter as shown in a representative plot. Gatings for KIR subsets were replicated in all the necessary combinations.

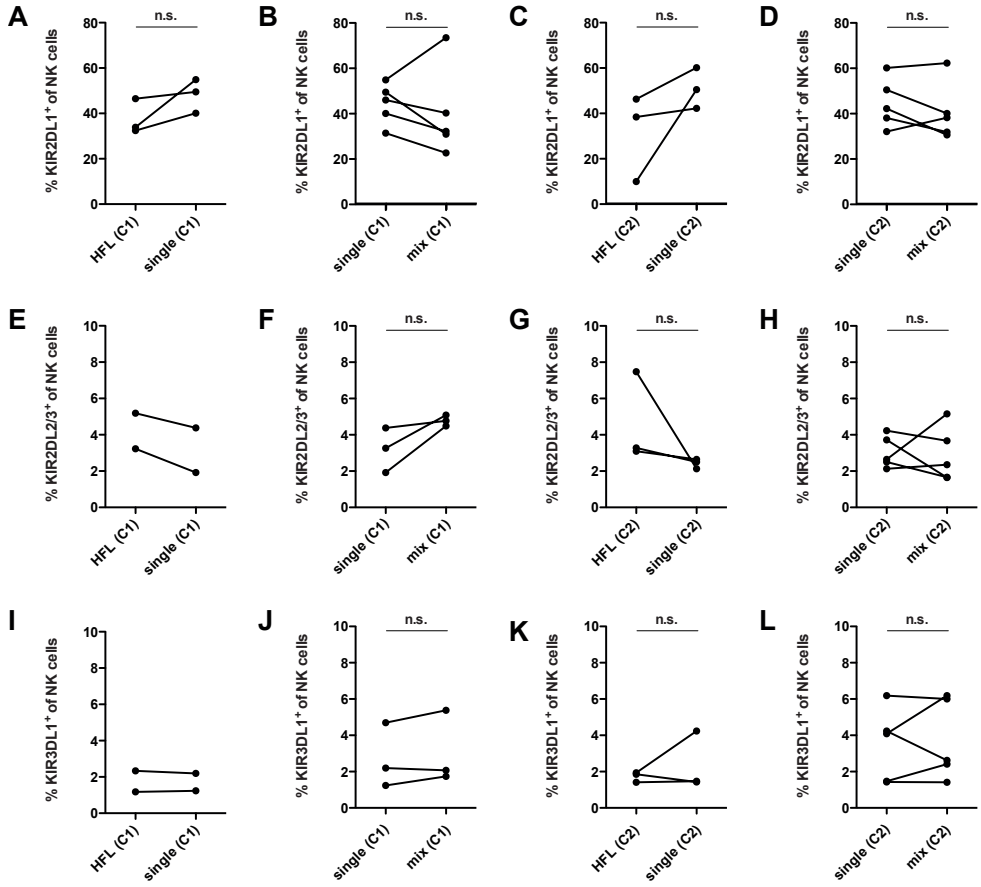

Figure S2, related to Figure 2. KIR frequencies at steady state represent original donor values and do not change in presence of non-cognate HLA in trans

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix).

(A to L) Mean percentage per experiment of single KIR+ liver NK cells as derived from total NK cells of huNSG mice.

(A to D) KIR2DL1+ NK cells of HLA-C1 single reconstituted huNSG mice as compared to HLA-C1 donor HFL NK cells (A) or HLA-C1 donor derived cells from mixed reconstituted huNSG mice (B) and conversely for HLA-C2 single reconstituted huNSG mice to HLA-C2 donor HFL (C) or HLA-C2 donor derived cells from mixed reconstituted huNSG mice (D).

(E to H) KIR2DL2/3+ NK cells of HLA-C1 single reconstituted huNSG mice as compared to HLA-C1 donor HFL NK cells (E) or HLA-C1 donor derived cells from mixed reconstituted huNSG mice (F) and conversely for HLA-C2 single reconstituted huNSG mice to HLA-C2 donor HFL (G) or HLA-C2 donor derived cells from mixed reconstituted huNSG mice (H).

(I to L) KIR3DL1+ NK cells of HLA-C1 single reconstituted huNSG mice as compared to HLA-C1 donor HFL NK cells (I) or HLA-C1 donor derived cells from mixed reconstituted huNSG mice (J) and conversely for HLA-C2 single reconstituted huNSG mice to HLA-C2 donor HFL (K) or HLA-C2 donor derived cells from mixed reconstituted huNSG mice (L).Data in are pooled data from at least three independent experiments. Dots represent mean of population in separate experiments, paired t test, n.s., not significant.

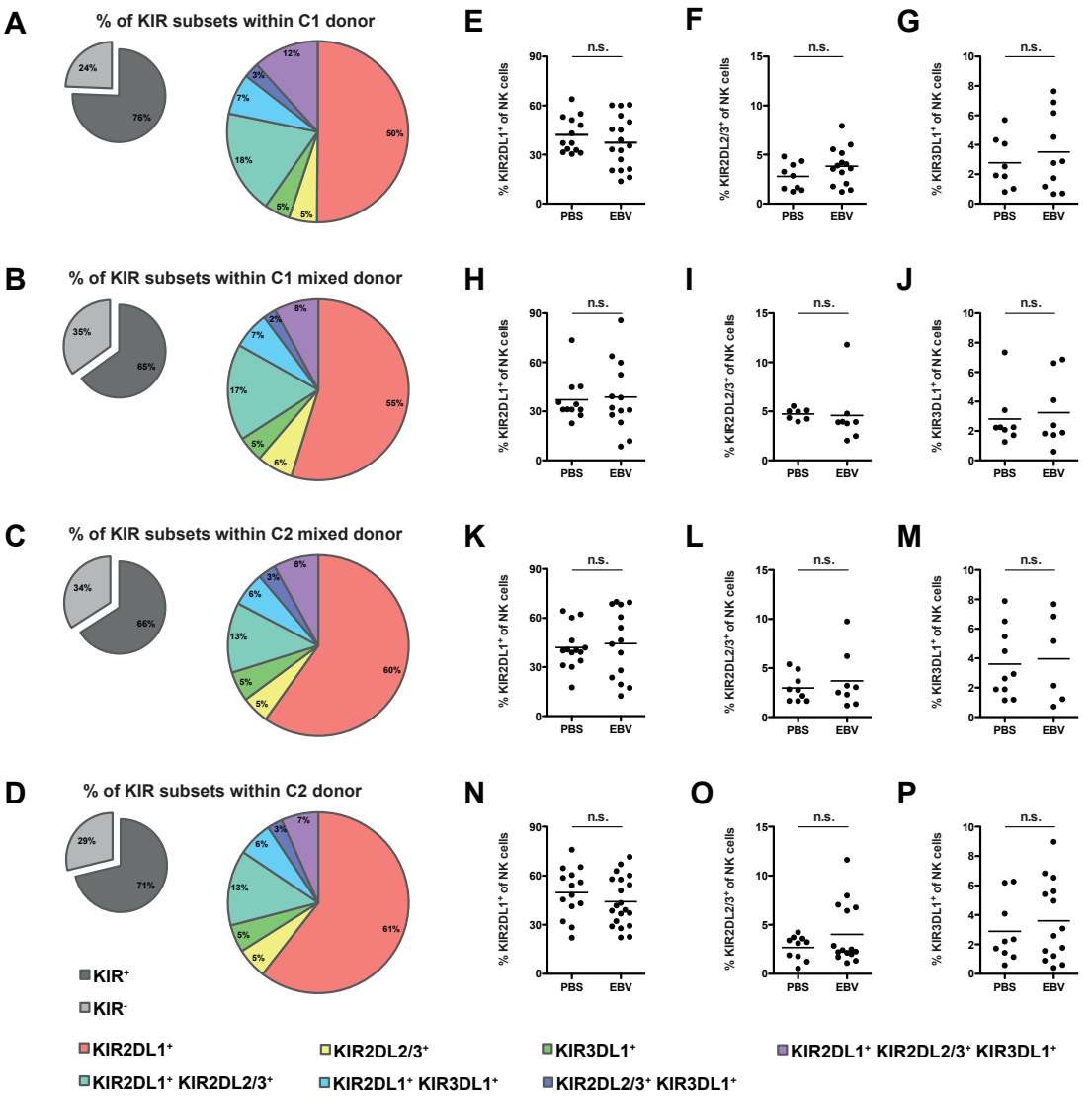

Figure S3, related to Figure 3. Infection with EBV does not lead to skewing of KIR repertoire

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix). Grey pie charts depict the ratio of KIR$^+$ to KIR$^-$ NK cells and colored pie charts represent the indicated KIR$^+$ NK cell subsets.

(A to D) Subsets of KIR expressing NK cells as percentages of total KIR$^+$ NK cells in liver of EBV infected huNSG mice.

(E to P) Frequency of single KIR$^+$ NK cells in liver of huNSG at endpoint. Comparison of PBS controls to EBV infected animals for frequency of KIR2DL1$^+$, KIR2DL2/3$^+$ or KIR3DL1$^+$ NK cells in homozygously reconstituted huNSG mice with HLA-C1 donor (E to G), mixed donor reconstituted huNSG mice with cells derived from HLA-C1 donor (H to J) or HLA-C2 donor (K to M) and homozygously reconstituted huNSG mice with HLA-C2 donor (M to P).

Data are pooled data from at least three independent experiments. n=8-15 mice per group, bar represents mean in relevant graphs, unpaired t test, n.s., not significant.

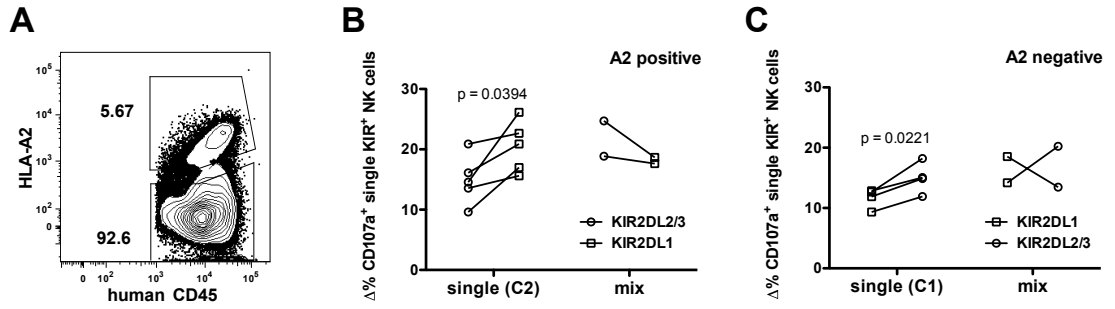

Figure S4, related to Figure 4. No titrating effect of non-cognate MHC class I on human NK cell education.

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1 or C2) or reconstituted with both the same HLA-C1 and -C2 donor disparate for HLA-A2. Representative experiment with unequal reconstitution in mixed huNSG mice.

(A) Representative dot plot showing overall human leukocyte reconstitution derived from HLA-C1 donor (HLA-A2 negative) and HLA-C2 donor (HLA-A2 positive) in mixed donor reconstituted huNSG mice (mix).

(B and C) Degranulation of single KIR-positive NK cells after incubation of K562 cells with splenocytes from huNSG mice reconstituted with HLA-C1 homozygous and/or HLA-C2 homozygous donors from the same experiment as shown in (A). Single KIR-positive NK cells were gated in both single and mixed reconstituted huNSG mice on cells derived from (B) HLA-C2 donor (A2 positive) or from (C) HLA-C1 donor (A2 negative). Data were analyzed by paired t tests. n.s., not significant.

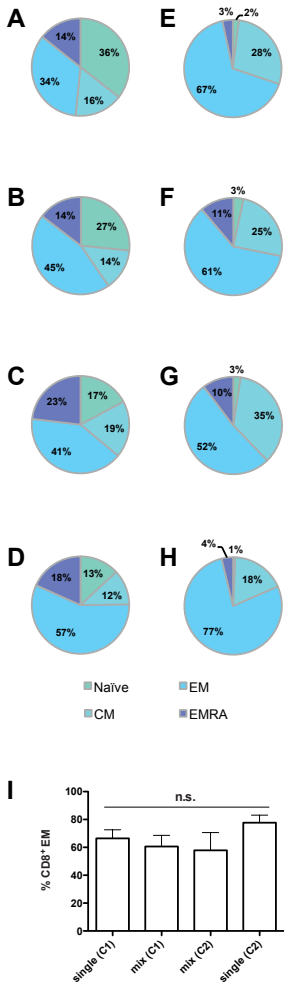

Figure S5, related to Figure 6. No change in T cell response of different reconstitutions

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix).

(A to I) Frequencies of T cell subsets in spleens of huNSG mice at experimental endpoints.

(A to D) $CD8^+$ T cell subsets in PBS control huNSG mice single reconstituted with homozygous HLA-C1 donor (A), HLA-C2 donor (D) or mixed donor reconstituted huNSG mice with T cells derived from HLA-C1 donor (B) and derived from HLA-C2 donor (C).

(E to H) $CD8^+$ T cell subsets in EBV infected huNSG mice single reconstituted with homozygous HLA-C1 donor (E), HLA-C2 donor (H) or mixed donor reconstituted huNSG mice with T cells derived from HLA-C1 donor (F) and derived from HLA-C2 donor (G).

(I) Comparison of frequencies of effector memory $CD8^+$ T cells in EBV infected single and mixed reconstituted huNSG mice at experimental endpoints in spleen.

Data are pooled data from at least two independent experiments. n=5-8 mice per group, mean ± SEM, unpaired t test, n.s., not significant.

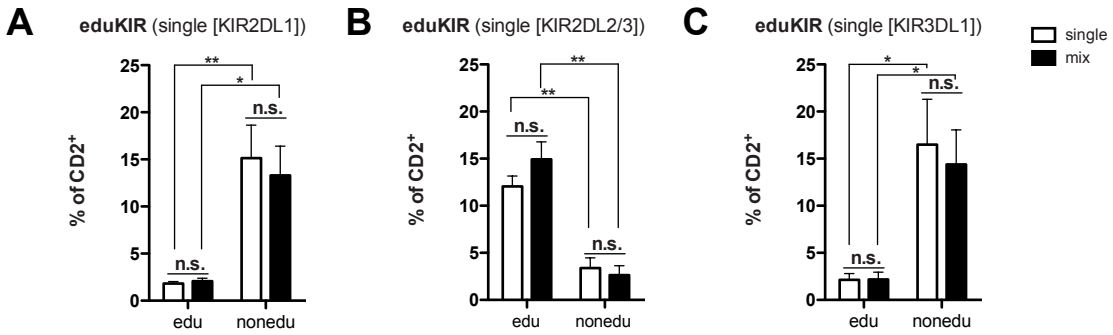

Figure S6, related to Figure 7. No increase in CD2⁺ NK cells in mixed donor reconstituted huNSG mice

HuNSG mice were single reconstituted with donors homozygous for HLA-C and -B allotypes (HLA-C1, -C2 and -Bw4) or reconstituted with two donors disparate for allotype and HLA-A2 expression (mix). According to single reconstituted allotypes, eduKIR denotes educated NK cell subset gated on cells derived from donor used for single reconstitution in both single and mixed reconstituted huNSG mice, respectively.

(A to C) Percentage of CD2⁺ cells in educated (edu) compared to non-educated (nonedu) NK cells in liver of huNSG mice (A) educated on HLA-C2 (KIR2DL1), HLA-C1 (KIR2DL2/3) (B) and HLA-Bw4 (KIR3DL1) (C).

Data are pooled data from at least two independent experiments. n=5-10 mice per group, mean ± SEM, ANOVA with Bonferroni correction and unpaired t test, n.s., not significant, *$p < 0.05$.

3.9 SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Table S1. Overview of fluorescently labeled antibodies

Molecule	Clone	Fluorophore	Company
CD107a	H4A3	FITC	BD Biosciences
NKp46	9E2	APC	BD Biosciences
KIR2DL1/S1	EB6B	PE Cy7	Beckman&Coulter
KIR2DL2/L3	GL183	PE Cy5.5	Beckman&Coulter
NKG2A	Z199	PE	Beckman&Coulter
CD14	M5E2	BV510	BioLegend
CD16	3G8	APC Cy7	BioLegend
CD19	HIB19	BV510	BioLegend
CD2	RPA-2.10	FITC	BioLegend
CD3	OKT3	BV510	BioLegend
CD3	OKT3	BV785	BioLegend
CD4	RPA-T4	BV510	BioLegend
CD4	RPA-T4	APC Cy7	BioLegend
CD45	HI30	BV605	BioLegend
CD45RA	HI100	BV510	BioLegend
CD57	HCD57	Pacific Blue	BioLegend
CD8	SK1	PerCP	BioLegend
HLA-A2	BB7.2	Pacific Blue	BioLegend
HLA-A2	BB7.2	FITC	BioLegend
HLA-A2	BB7.2	PE	BioLegend
HLA-DR	L243	PE Cy7	BioLegend
KIR2DL1/S1	HP-MA4	FITC	BioLegend
KIR2DL2/L3	DX27	PE	BioLegend
KIR3DL1	DX9	Alexa Fluor 700	BioLegend
CD19	SJ25-C1	PE Texas Red	invitrogen
CCR7	150503	PE	R&D Systems

CHAPTER FOUR

INTERACTION OF NATURAL KILLER CELLS WITH PLASMACYTOID DENDRITIC CELLS

HUMAN NATURAL KILLER CELLS FORM ACTIVATING SYNAPSES WITH PLASMACYTOID DENDRITIC CELLS WITHOUT KILLING THEM

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4.1 ABSTRACT

Resting natural killer (NK) cells require activation by dendritic cells (DCs) to achieve their full protective functions during viral infections. While the interaction of human NK cells with conventional DCs has been investigated in great detail, the stimulation of NK cells by plasmacytoid DCs (pDCs) has been less explored. Here, we report that pDCs form immunological synapses with NK cells. These are enriched in NK cell derived f-actin and type I interferon (IFN) receptors. Furthermore, NK cells are activated through this interaction with IFN receptor signaling via STAT1 phosphorylation at the synapse. However, despite the immunological synapse between pDCs and NK cells displaying hallmarks of an activating NK cell synapse, pDCs are not killed in this interaction. Thus, the two innate leucocyte populations, pDCs and NK cells might sustain anti-viral functions by their interaction early during infections.

4.2 INTRODUCTION

Natural killer (NK) cells have been originally recognized for their cytotoxic function against transformed and infected cells (Herberman et al., 1975a; Kiessling et al., 1975). Different developmental stages of this innate lymphocyte lineage can be found outside the bone marrow and these seem to fulfill specific functions in their respective anatomical locations in humans (Ferlazzo and Münz, 2004). $CD56^{bright}CD16^{-}$ NK cells constitute the main NK cell subpopulation in human secondary lymphoid tissues (Fehniger et al., 2003; Ferlazzo et al., 2004b). They produce mainly cytokines after cytokine stimulation at these sites and seem to polarize as well as mature dendritic cells (DCs) to facilitate the priming of Th1 responses (Morandi et al., 2006; Moretta, 2002). These $CD56^{bright}$ NK cells can be matured into $CD56^{dim}CD16^{+}$ NK cells (Ferlazzo et al., 2004b; Huntington et al., 2009; Romagnani et al., 2007), which predominate in the blood and respond to infected or transformed target cell recognition by cytokine production and cytotoxicity (De Maria et al., 2011; Vivier et al., 2011). Upon further differentiation they acquire more and more killer immunoglobulin-like receptor (KIR) expression (Björkström et al., 2010). Thus human NK cells home to different tissues during their differentiation and fulfill different functions at these sites.

Preferentially in secondary lymphoid tissues, NK cells can be activated by dendritic cells (DCs) in order to fulfill a barrier function against invading pathogens at these sites and to polarize T cell responses (Ferlazzo et al., 2004a; Lucas et al., 2007; Strowig et al., 2008). In this interaction different DC-derived cytokines mediate the NK cell activation. While IL-12 stimulates cytokine production, IL-15 promotes NK cell survival and proliferation, while type I interferons (IFN-α/β) enhance NK cell cytotoxicity (Brilot et al., 2007; Ferlazzo et al., 2004a; Gerosa et al., 2005; Strowig et al., 2008). In humans, this type I IFN can come from plasmacytoid DCs (pDCs), in particular after their stimulation with single-stranded RNA (toll-like receptor 7 [TLR7] recognition) or with unmethylated DNA (TLR9 recognition) (Gilliet et al., 2008; Meixlsperger et al., 2013). Thus, cytokines play a prominent role in the cross-talk between NK cells and DCs, particularly type I IFN in the interaction with pDCs.

NK cells mediate their cellular interactions via immunological synapses (IS) (Orange, 2008). The phenotypic characteristics of these synapses determine the outcome of the interaction. Activating IS, which lead to the killing of the conjugated target cells, are characterized by actin polymerization at the IS side of the conjugated NK

cell, polarization of the microtubule organizing center (MTOC) of the NK cell to the interface and directed secretion of cytotoxic granules and IFN-γ into the synaptic cleft (Brown et al., 2012). In contrast, when inhibitory interactions dominate the synapse between NK cells and target cells, the IS disassembles faster, and shows none of the above mentioned polarization events. Therefore, NK cells stabilize activating synapses with transformed and infected cells by cytoskeletal remodeling to deliver directed lethal blows to these targets.

In contrast to these classical categories of NK cell synapses we present here the characterization of the immunological synapse between plasmacytoid DCs and NK cells. Although NK cells polymerize actin at the interface with pDCs, polarize the type I IFN receptor to the resulting IS and get activated through this interaction, pDCs are not killed in the process.

4.3 RESULTS

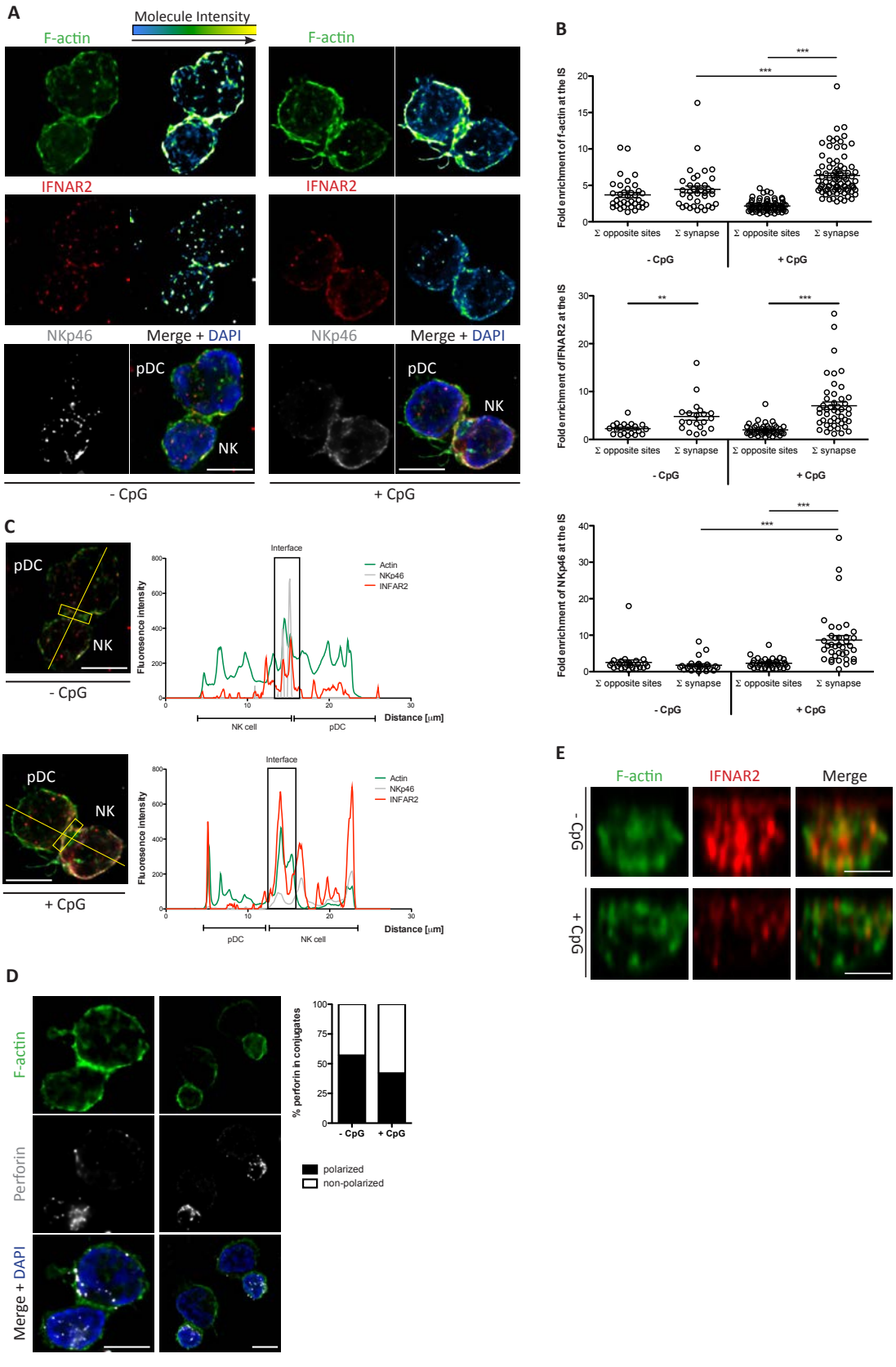
4.3.1 Human NK cells and pDCs form immunological synapses with polarization of actin and the type I IFN receptor in NK cells

It has been previously shown that conventional DCs can form regulatory immunological synapses with autologous NK cells and that this leads to the efficient activation of these innate lymphocytes (Barreira da Silva et al., 2011; Brilot et al., 2007). Furthermore, plasmacytoid DCs as main type I IFN producers are known to be potent activators of NK cell cytotoxicity (Gerosa et al., 2005; Trinchieri, 1989; Trinchieri and Santoli, 1978). Therefore, we investigated whether pDCs were also capable of forming conjugates and structured immunological synapses with NK cells. To study this, we stimulated purified human pDCs with the toll-like receptor 9 (TLR9) agonist CpG and cultured them or unstimulated control cells with resting autologous NK cells for 20 min. We then analyzed the formation of immunological synapses by immunofluorescence (Fig. 1A). Both CpG-stimulated and unstimulated pDCs were able to form conjugates with NK cells. However, only CpG-stimulated pDCs were able to induce a significant polymerization of filamentous actin (f-actin) as well as of the NK cell specific marker NKp46 at the synapse. In contrast the type I IFN receptor subset IFN-α/βR2 was significantly enriched at the synapses of both unstimulated and CpG-stimulated pDCs with NK cells (Fig. 1B). However, IFN-α/βR2 was more significantly enriched at immunological synapses of CpG-stimulated pDCs with NK cells. To determine which interaction partner contributed the accumulation of f-actin and IFN-α/βR2 at the cellular interface, staining for NKp46 was overlayed with stainings for the molecules in question. In both cases, colocalization of fluorescence intensity was on the NK cell derived side of the immunological synapse (Fig. 1C). Analysis of the peripheral and central supramolecular activation clusters (pSMAC, cSMAC) showed accumulation of f-actin in a ring-like structure along the plane of interaction between NK cells and pDCs with the IFN-α/βR2 interspersed between the f-actin (Fig. 1E). Although the immunological synapse between pDCs and NK cells showed with NK cell derived f-actin polarization the hallmarks of a cytotoxic NK cell synapse, we did not observe polarization of cytotoxic granules to the interface. For example, immunofluorescence staining for perforin showed random distribution of this cytolytic protein within the conjugated NK cells (Figure 1D). These observations pose the question which function the observed synapse between pDCs and NK cells has. To that end we investigated downstream signaling through the IFN-α/βR receptor, which relies on the JAK-STAT pathway (Platanias, 2005). Conjugates were stained for

phosphorylation of tyrosine 701 on the STAT1 protein (Fig. 2A). Quantification of phosphorylated STAT1 demonstrated that it accumulated to a greater extent in NK cells at synapses of unstimulated pDCs (Fig. 2B,C), indicating a possible function of the synapse, to concentrate and direct type I IFN signaling towards NK cells in the absence of strong IFN-α secretion.

Figure 1. The formation of an activating synapse between stimulated pDCs and resting NK cells leads to the accumulation of f-actin and IFN-α/βR2 at the synapse.

(A) pDCs with or without CpG stimulation overnight were incubated with resting NK cells for 20min and fixed. F-actin was stained with bodipy conjugated phallacidin (green), nuclear DNA was stained with DAPI (blue), anti-IFN-α/βR2 (red) and anti-NKp46 (grey) antibodies were used for surface markers. (B) The fluorescence intensity of the opposite sides in both cells was summed up and plotted against the fluorescence intensity at the synapse to plot the accumulation of f-actin and IFN-α/βR2 and NKp46 at the synapse. (C) The fluorescence intensity was plotted along a trajectory through both cells with the synapse indicated by boxes. (D) Synapses were analyzed through their volume and the z-plane was reconstructed to visualize the pSMAC and cSMAC. (E) pDCs with or without CpG stimulation overnight were incubated with resting NK cells for 20min and fixed. F-actin was stained with bodipy conjugated phallacidin (green), nuclear DNA was stained with DAPI (blue) and anti-perforin (grey) was used to visualize cytotoxic granules. Polarization of perforin to the IS was stated as percentage of all evaluated conjugates where accumulation was observed at the synapse. The data are representative of or summarize three experiments.


A
Molecule Intensity
F-actin
F-actin
IFNAR2
IFNAR2
NKp46
Merge + DAPI
NKp46
Merge + DAPI
pDC
NK
pDC
NK
- CpG
+ CpG
B
Fold enrichment of f-actin at the IS
Fold enrichment of IFNAR2 at the IS
Fold enrichment of NKp46 at the IS
Σ opposite sites
Σ synapse
Σ opposite sites
Σ synapse
- CpG
+ CpG
C
pDC
NK
- CpG
Interface
Actin
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INFAR2
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Distance [µm]
NK cell
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pDC
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+ CpG
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Actin
NKp46
INFAR2
Fluorescence intensity
Distance [µm]
pDC
NK cell
D
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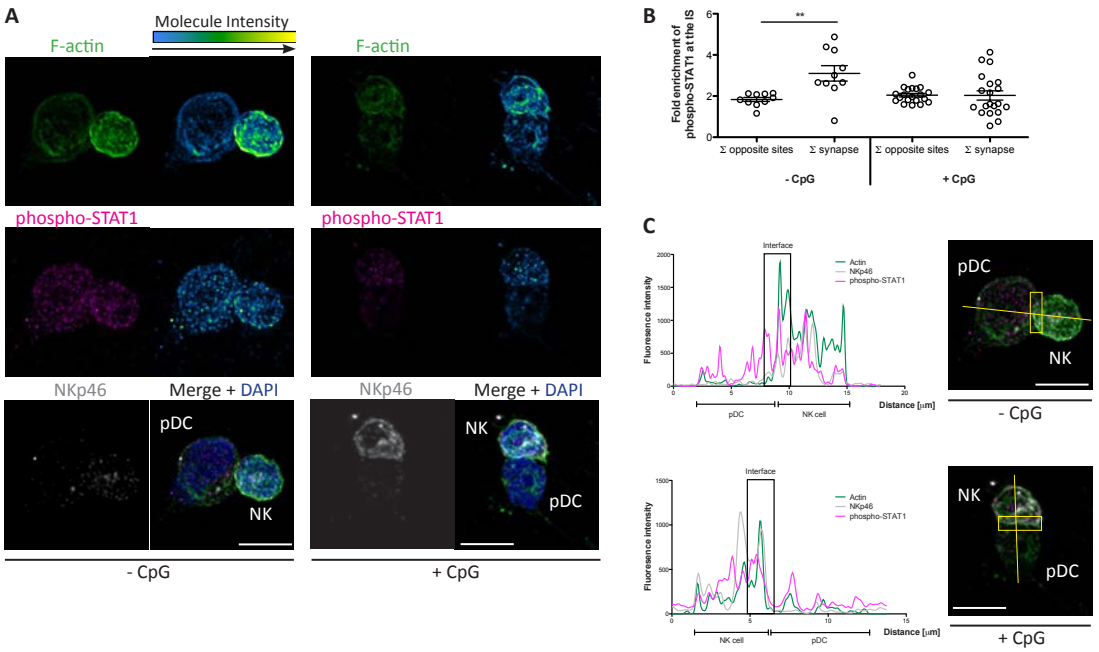

Figure 2. The formation of an activating synapse between unstimulated pDCs and resting NK cells leads to the increased signaling via the JAK-STAT pathway at the IS.

(A) pDCs with or without CpG stimulation overnight were incubated with resting NK cells for 20min and fixed. F-actin was stained with bodipy conjugated phallacidin (green), nuclear DNA was stained with DAPI (blue), anti-phospho-STAT1 (magenta) and anti-NKp46 (grey) antibodies were used for surface markers. (B) The fluorescence intensity of the opposite sides in both cells was summed up and plotted against the fluorescence intensity at the synapse to plot the accumulation of f-actin and phospho-STAT1 at the synapse. (C) The fluorescence intensity was plotted along a trajectory through both cells with the synapse indicated by boxes. The data are representative of or summarize three experiments.

4.3.2 Interaction with pDCs leads to phenotypic activation of human NK cells

It has been demonstrated that monocyte-derived DCs are capable of notably increasing surface expression of the activation marker CD69 on NK cells (Brilot et al., 2007; Draghi et al., 2007; Vitale et al., 2004). Another recognized marker for NK cell activation is their expression of HLA-DR upon stimulation (Bozzano et al., 2009; Ferlazzo et al., 2003; Ingram et al., 2009). Therefore, we decided to monitor the surface expression of these markers as readout for NK cell activation by pDCs. Isolated NK cells were incubated overnight on their own or with unstimulated or CpG-stimulated autologous pDCs under transwell or co-culture conditions. The read out by flow cytometry showed that HLA-DR surface levels were evidently upregulated on NK cells in co-cultures of both unstimulated and CpG-stimulated conditions (Fig. 3A). However, HLA-DR molecules on NK cells were more detectable after co-culture with CpG-stimulated pDCs and could result from expression by NK cells or trogocytosis from pDCs. Similarly, CD69 on NK cells only showed a significantly increased expression in co-cultures between CpG-stimulated pDCs and NK cells. In contrast, pDCs did not upregulate these surface molecules upon co-culture with NK cells (Fig. 3B). These findings indicate that pDCs, especially after CpG stimulation, activate NK cells, and that this activation occurs more efficiently in conjugates of pDCs with NK cells.

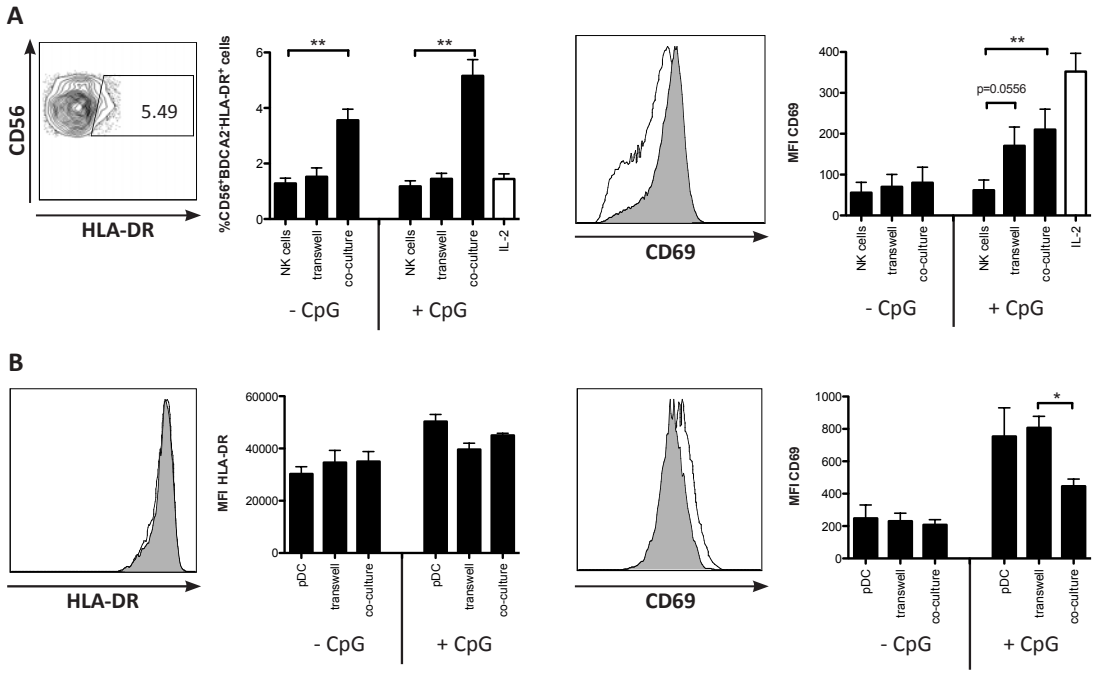

Figure 3. NK cells show phenotypic maturation upon co-culture with pDCs.

Co-cultures of resting NK cells and pDCs with or without CpG stimulation were set up. Cells were cultured with direct cell-contact or in transwell plates. (A) NK cells were analysed by staining of surface markers with anti-HLA-DR and anti-CD69 antibodies for expression and upregulation. (B) pDCs were analysed by staining of surface markers with anti-HLA-DR and anti-CD69 antibodies for expression and upregulation. The data are representative of or summarize three experiments.

4.3.3 pDCs increase NK cell cytotoxicity

In addition to the phenotypic NK cell activation during co-culture with pDCs, we also addressed if NK cell cytotoxicity can be stimulated by pDCs. pDC induced NK cell cytotoxicity has been reported to be cell-contact independent (Gerosa et al., 2005) and also in our experiments transwell separation did not alter the increased NK cell cytotoxicity after co-culture with pDCs (data not shown). Therefore, we incubated NK cells with different doses of pDC-derived medium. After 4 days of incubation, the NK cells were used in a killing assay against the erythroleukemic K562 cell line to determine NK cell cytotoxicity against these target cells with high susceptibility to NK cell lysis. We observed enhanced cytotoxicity of NK cells that were conditioned with CpG-stimulated pDC supernatant (Fig. 3C). We determined the IFN-α concentrations in the used pDC supernatants and found that the IFN-α concentrations in these correlated with specific K562 lysis by supernatant exposed NK cells (Fig. 3D). Furthermore, a threshold value of 1000pg IFN-α was necessary to enhance cytotoxic responses of NK cells significantly (Fig. 3E). These experiments indicate, that soluble type I IFNs secreted from pDCs are able to stimulate NK cell cytotoxicity.

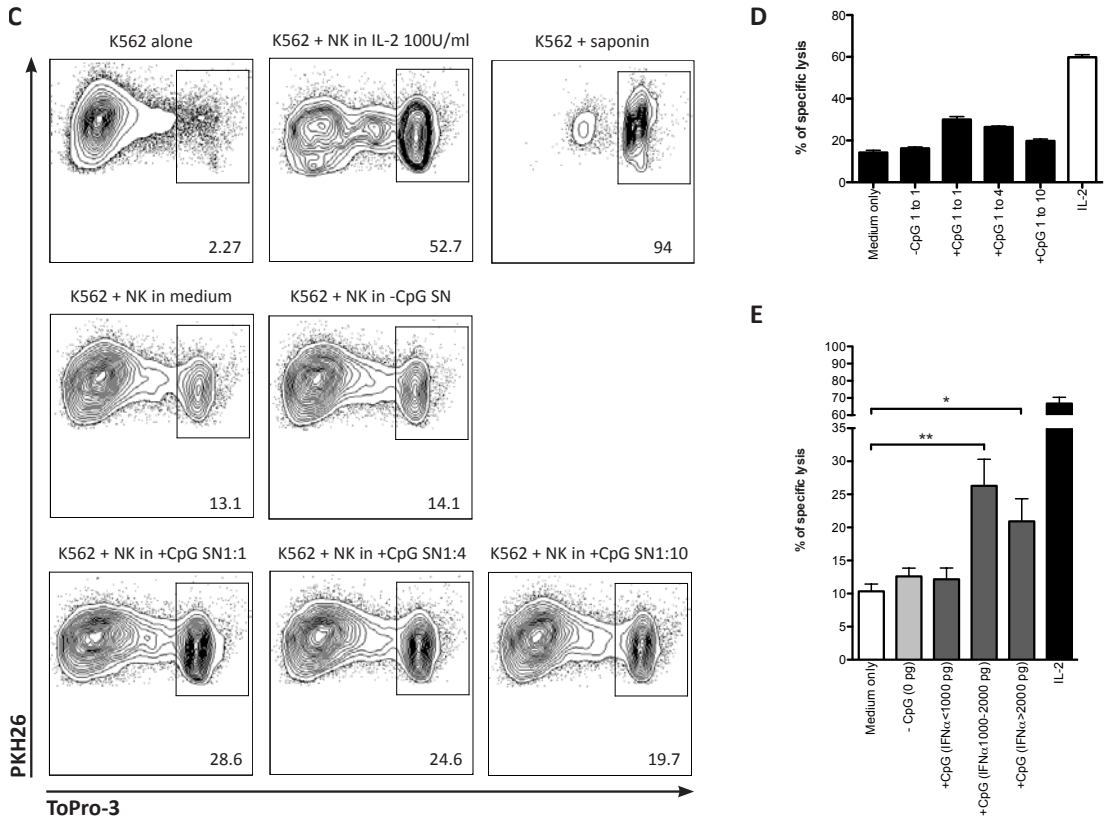

Figure 3. NK cells show functional maturation upon co-culture with pDCs.

Co-cultures of resting NK cells and pDCs with or without CpG stimulation were set up. Cells were cultured with direct cell-contact or in transwell plates. (C) NK cells were incubated for 4 days with supernatant derived from pDCs cultured overnight with or without CpG stimulation. After stimulation of the NK cells they were incubated for 6h with the target cell line K562, pre-stained with PKH26. NK cell cytotoxicity was assessed by quantification of dead cells using To-Pro-3-iodide. (D) Specific lysis was determined on the basis of the spontaneous lysis of K562 cells on their own and total lysis upon saponin incubation. (E) Percent of specific lysis shows a positive correlation to the amount of IFN-α present in the supernatant of pDCs. The data are representative of or summarize three experiments.

4.3.4 pDCs are not killed by NK cells

We next wanted to analyze the reciprocal interaction in pDC conjugates with NK cells. Accumulation of f-actin at the cell-cell contact site in NK cells is a hallmark of cytotoxic NK cell synapses (Carpén et al., 1983; Katz et al., 1982) and non-activated conventional DCs can be killed by NK cells (Ferlazzo et al., 2002; Jungraithmayr et al., 2013). Therefore, we examined the impact of NK cell presence on pDC survival. pDCs were cultured overnight alone, in transwell or in co-culture with autologous NK cells. When looking at the percent of surviving pDCs, a significant difference with increased pDC survival could be observed between unstimulated pDCs alone and those that had been cultured in the presence of NK cells. In the case where CpG was added to the cells, survival was high overall since this agonist acts as a proliferation and survival stimulus for pDCs (Fig. 4A). Since direct cell-contact was not a requirement for pDC survival in the co-cultures, we tested the influence of NK cell supernatant on the survival of pDCs. We could confirm that in the presence of soluble stimuli provided by the NK cells, the pDCs showed increased survival (Fig. 4B). Also total cell counts of pDCs after NK cell supernatant transfer showed a slightly better survival of pDCs (Fig. 4C). However, we were not able to determine the NK cell secreted factor that promotes pDC survival. IL-3, GM-CSF and Flt3-L were not produced by NK cells at significant levels under our culture conditions (Fig. 4D).

Instead, expression of TNF-α and IFN-γ could be detected from overnight co-cultures of NK cells with pDCs and pDC supernatants (Fig. 4E). Further analysis demonstrated, however, that the bulk TNF-α originated from stimulated pDCs. Further evidence for the non-cytolytic nature of the IS between pDCs and NK cells came from ultrastructural investigations by transmission electron microscopy (TEM), in which the synaptic cleft between these innate leucoytes was quantified. Distances ranged between 90-160nm (median = 114nm, Fig. 4F) reminiscent of the range measured for regulatory synapses of NK cells with conventional DCs (Barreira da Silva et al., 2011). Therefore, pDCs are not getting killed in the interaction with NK cells, although they form an immunological synapse with these innate lymphocytes that has hallmarks of an activating NK cell synapse.

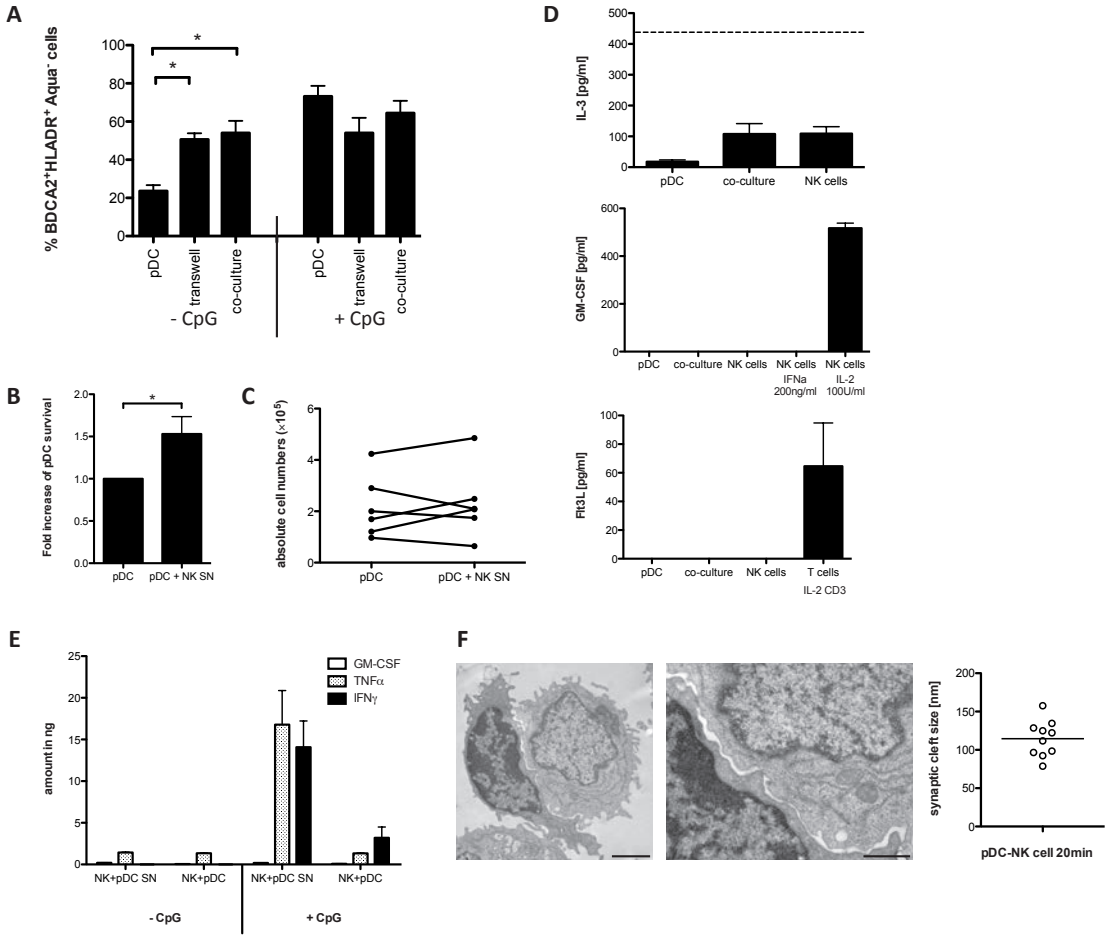

Figure 4. NK cells aid pDC survival in absence of a TLR agonist.

(A) Unstimulated and CpG-stimulated pDCs were incubated overnight alone or with NK cells in transwell or co-culture. The percent of BDCA2$^+$ HLA-DR$^+$ Aqua$^-$ cells was used as the readout for pDC survival. (B) Supernatants from allogeneic NK cells were used to stimulate pDCs overnight. Survival was quantified as fold increase compared to pDCs incubated with plain medium. (C) Supernatants from allogeneic NK cells were used to stimulate pDCs overnight. Survival was evaluated by counting absolute cell numbers. (D) ELISAs for IL-3, GM-CSF and Flt3L were run on pDC, co-culture and NK cell supernatants to find the cytokine promoting pDC survival. (E) ELISAs for GM-CSF, TNF-α and IFN-γ were run on supernatants of NK cells co-cultured with pDCs or pDC supernatants to determine the cytokine expression profile of NK cells. (F) Overnight CpG stimulated pDCs were incubated with resting NK cells for 20min, fixed and processed for TEM. The synaptic cleft between the two cells was quantified by measuring the interspace. Scale bars represent 2µm and 1µm for left to right. The presented data are representative of or summarize a minimum of two experiments with exception of TEM images.

4.4 CONCLUSIONS

pDCs readily form immunological synapses with NK cells. These are stabilized by f-actin polymerization from the NK cell side. At least phenotypic NK cell activation is facilitated through this synapse, while stimulation of NK cell cytotoxicity is also transferred with NK cell supernatant, presumably primarily via type I IFNs. Nevertheless, this IFN signaling is optimized, especially under IFN limiting conditions at the synapse and, accordingly, STAT1 phosphorylation can be observed at the synapse. This optimized IFN signaling might be especially important in tissues, where secreted type I IFN might be easily bound and consumed by neighboring cells. Thus NK cells form activating synapses with pDCs. However, pDCs are not killed in this interaction, but even survive slightly better upon encountering NK cells. Thus it would be interesting if these two innate leucocyte populations also support each other during early phases of infections with pathogens in humans.

4.5 MATERIAL AND METHODS

Antibodies and labeling reagents

Directly labeled anti-CD56 (clone B-159), -NKp46 (9E27NKp46), -CD69 (FN50) and -CD16 (3G8) antibodies were purchased from BD Bioscience. Anti-HLA-DR (L242), anti-perforin (dG9) were from Biolegend and anti-BDCA2 (AC144) was from Miltenyi Biotec. Rabbit anti-IFN-α/βR2 was from Abcam and rabbit anti-Phospho-STAT1 pTyr701 antibody (F.747.9) was from Thermo Scientific, both unconjugated. Cells labeled with unconjugated antibodies were stained with Alexa Fluor 555- conjugated donkey anti-rabbit IgG or Alexa Fluor 647-conjugated goat anti-mouse IgG from Invitrogen. Control antibodies for stainings were normal rabbit IgG (Jackson Immunoresearch). Live and dead cell staining in flow cytometry experiments was performed with an aqua fluorescent reactive dye from Invitrogen.

Plasmacytoid DCs and NK cells

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte concentrates (Zürich Blood Center) by density-gradient centrifugation on Ficoll/Hypaque (GE Healthcare). $BDCA4^+$ plasmacytoid DCs were isolated from PBMCs by positive magnetic cells separation (Miltenyi Biotec). Isolated pDCs were cultured overnight in R2 (RPMI 1640 (GIBCO) supplemented with 2% heat-inactivated pooled human AB serum (Invitrogen)) and gentamicin (10mg/ml, Invitrogen), in the presence or absence of CpG2216 (10**µ**g/ml, Invivogen). NK cells were isolated from $BDCA4^-$ fractions by negative selection using the NK cell isolation kit II (Miltenyi Biotec). If necessary NK cells were frozen down or cultured in R5 (RPMI 1640 supplemented with 5% heat-inactivated pooled human AB serum) and gentamicin (10mg/ml). Activated NK cells were stimulated with 100U/ml of IL-2 (Peprotech) for 4 days, when not specified otherwise.

Co-cultures

Isolated pDCs and NK cells were cultured either alone or in the presence of each other in co-cultures overnight. For the transwell conditions a HTS Transwell 96 well permeable support, pore size 0.4 µm from Corning Life Science was used.

K562 cells

K562 cells were maintained in RPMI 1640 medium supplemented with gentamicin (10mg/ml) and 10% FCS (Sigma). Cell cultures were kept in a water-saturated atmosphere with 5% CO_2 and 37°C.

Human plasmacytoid DC and resting NK cells conjugate assays

Resting NK cells were mixed with unstimulated or CpG-stimulated autologous plasmacytoid DCs at a ratio of 2:1 in 20µl of RPMI 1640 without serum, prior to being pelleted shortly at 10'000g. The cells were allowed to conjugated for 20min at 37°C. The cells were resuspended in RPMI 1640 and centrifuged onto polylysine-coated 1.5mm coverslips for immunofluorescence processing. Alternatively co-cultures were fixed and treated for TEM analysis.

Immunofluorescence microscopy and analysis

Cells on slides were fixed in 3% PFA for 20min at 4°C. Cells were permeabilized with 0.01%Triton-X for 1 min at room temperature. Cells were then incubated with the Image-iT FX signal enhancer (Invitrogen) and stained with the indicated antibodies followed by the appropriate secondary reagent. All washes were performed in PBS supplemented with 1% fish skin gelatin (Sigma-Aldrich) and 0.02% saponin (Sigma-Aldrich). After staining, slides were counterstained with DAPI and mounted with Prolong gold anti-fade reagent (Invitrogen). Slides were visualized through a ×100 1.4 NA oil immersion lens with an inverted Leica LX microscope and a Leica DFC 350 FX camera or an inverse confocal CLSM Leica SP8 gated STED microscope. Serial optical sections (0.2µm or 0.17µm; 17-64 sections) were acquired for all labelings. Images were deconvolved using Huygens software 1.1.4 (Montpellier RIO Imaging). Fold enrichment values were determined by evaluation of conjugates in randomly selected fields in at least 3 separate experiments. pDC/resting NK cell conjugates were identified using: (a) chromatin density, in which DAPI staining stains brightly condensed and round-shaped nuclei of NK cells as opposed to less dense pDC nuclei; (b) staining of the NK cell surface marker NKp46; and (c) f-actin staining, by which cell-cell interaction can be properly visualized, cytoplasm can be identified and time of interactions can be selected on the basis of f-actin polymerization at the synapse. Quantification of molecules at the synapse

in conjugates was done after image analysis with Image J software version 1.45s (NIH). We measured the enrichment of a molecule at the contact site of each conjugate compared with the distribution of the same molecule in the area opposite to the contact site within the same cell. The fold enrichment was calculated as the average intensity per unit volume at the contact site or the sum of the opposite membranes across the contact site divided by the average intensity per unit volume of the entire cell. The values obtained at opposite sites of the cells were considered as baseline for statistics, and data represent the ratio of relative enrichment at the contact site to relative enrichment at the membrane across the contact site normalized to the values in single cells. Fluorescence intensity of different markers was measured across cells using Image J plot profile. Z-stack reconstruction to identify the distribution of molecules in pSMACs and cSMACs was performed on images acquired at the confocal microscope by rotating them so the entire contact site could be viewed. Polarization of perforin was evaluated by visually assessing the presence of granules at the IS. Images represent projections of acquired z-planes and scale bars are equivalent to 10μm.

Transmission Electron Microscopy and analysis

Samples were process at the Center for Microscopy and Image Analysis, UZH. Images were acquired using TEM Zeiss Leo 912 Omega microscope. Original magnifications were 7'400x and 17'500x from left to right. The synaptic cleft between NK cell-pDC conjugates was determined by measuring the distance between two cells using the line tool and ROI manager in Image J.

K562 and NK cell killing assay

NK cells were left untreated or incubated for 4 days with supernatant of unstimulated pDCs or different doses from CpG-stimulated pDCs. As positive control NK cells were incubated with 100U/ml of IL-2 and as a negative just with plain medium. K562 cells were prestained with the PKH26 dye (Sigma) and cultured with incubated NK cells at a ratio of 2:1 for 6h at 37°C in R10 (RPMI 1640 supplemented with 10% FCS) and gentamicin (10mg/ml). For assessing susceptibility of K562 cells to NK cell-mediated killing, K562 single cultures or K562/NK cell co-cultures were stained with To-Pro-3-iodide (Invitrogen).

ELISA

After overnight culture plasmacytoid DCs were collected and IFN-α levels in the supernatants were measured by ELISA for human Interferon-α MT1/2 (Mabtech). After overnight culture NK cells were collected and GM-CSF (Mabtech), IL-3 (abcam), Flt-3L (R&D Systems), TNF-α (Mabtech) and IFN-γ (Mabtech) were measured.

Flow Cytometry

Cells were acquired on a FACSCanto II or LSR II Fortessa (BD Biosciences) and all flow cytometry analysis were performed with FlowJo software (Tristar).

Statistics

Statistical analyses were performed with the Mann Whitney test, non-parametric and bicaudal. *P* values lower than 0.05 were considered significant. Plotted data are displayed as median ± interquartile ranges or means ± SEMs.

4.6 ACKNOWLEDGEMENTS

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CHAPTER FIVE

DISCUSSION

PART I – KIR REPERTOIRE AND EDUCATION

5.1 Novel humanized mouse model of two co-reconstituting donors

Taking advantage of mixed $CD34^+$ hematopoietic progenitor cell reconstituted NSG mice, we were able to investigate the influence of non-cognate HLA in trans on the NK cell compartment of the engrafting human immune system. This allowed us to address questions of KIR repertoire generation and NK cell education, which so far have been very difficult to experimentally address.

To the best of our knowledge, this is the first work studying the direct influence of cells from two engrafting human hematopoietic systems on each other. In the clinical setting of double-unit umbilical cord blood transplantations, only one donor prevails, leading essentially to single unit engraftment (Gutman et al., 2010; Hashem and Lazarus, 2015). This single-unit dominance seems to be mediated by co-transferred T cells and is without an obvious influence of NK cell interactions (Gutman et al., 2010; Tarek et al., 2015). Data from our reconstitutions show that mixed reconstituted huNSG mice were able to engraft all compartments of the immune system similar to single reconstituted mice (Strowig et al., 2009; 2010). Furthermore, we could demonstrate that both donors were represented at equal frequencies with regard to the main lymphocyte subsets. Thus, we established a model where two engrafted donors were able to develop and persist side-by-side.

5.2 Skewing of KIR repertoire under steady state and EBV-induced inflammatory conditions

Several studies have provided evidence that the KIR repertoire can be modulated to some extent by HLA class I expression (Shilling et al., 2003). Our data however showed that the KIR repertoire remained diverse after NK cell development in single as well as mixed reconstituted huNSG mice during steady state, with frequencies representing those found in the donor fetal liver tissue. This suggests that HLA only influences the repertoire marginally, in *cis* and/or *trans*, if at all. In addition, in HCMV-seropositive patients, a history of HCMV infection was found to leave the KIR repertoire visibly

skewed in later life (Béziat et al., 2013; Gumá et al., 2004). Hence, exposure to pathogens, in particular HCMV, is thought to be able to strongly influence the KIR repertoire of NK cells. Contrary to HCMV, the KIR repertoire found in huNSG mice remained unchanged during EBV-induced inflammatory conditions and continued to resemble a repertoire as found throughout steady state. Therefore, it seems that only the beta-herpesvirus CMV, but not other herpesviruses like EBV or HSV {Bjorkstrom:2011hp}, is adept at skewing the KIR repertoire in humans.

5.3 Education and disarming of huNSG mouse

Studying NK cell education has been challenging due to the lack of phenotypic markers. Solely upregulation of DNAM-1 and decreased expression of self-specific KIRs seem to characterize educated NK cells (Enqvist et al., 2015; van Bergen et al., 2013) whereas NK cell differentiation occurs independently of education by self-HLA ligands (Björkström et al., 2010). Thus, readouts are restricted to assays that explore the functional capacity of NK cells in order to determine their educational status (Höglund and Brodin, 2010). Furthermore, the precise cells mediating NK cell education, whether hematopoietic or stromal in nature, have not been conclusively identified (Bessoles et al., 2013). Although both have been implicated in influencing education, recent studies mainly support a favoring role of the hematopoietic system. This was exemplified in a study where the educating compartment was elucidated in more depth using a cohort of HLA-mismatched allogeneic hematopoietic cell donor transplants to identify the role of stromal versus hematopoietic cells. Educated donor NK cells were found within the first year after transplant and could be followed for three years thereafter. In HLA-mismatched transplants, education of NK cells was determined by the donor's HLA/KIR-ligands. Hence, suggesting that the educator was of hematopoietic cell origin (Haas et al., 2011). Furthermore, in mice with inducible MHC expression, it has clearly been shown that the hematopoietic compartment had a much bigger impact on education compared to the stromal cells (Ebihara et al., 2013). In addition, it has been reported that already the presence of low amounts of MHC-negative host cells could induce hyporesponsiveness in NK cells (Johansson et al., 1997). Together, these data indicate that the hematopoietic compartment is key in NK cell education and that the threshold for downregulating NK cell functionality in response to an educational signal is much more sensitive than through effector induction. In accordance with that, our data shows

that NK cells of single reconstituted mice are being educated in the presence of the educating HLA. However, in mixed reconstituted mice, we find that NK cell education is abrogated due to the absence of cognate HLA in trans. Therefore, in keeping with the findings mentioned above, our results from one as well as two engrafting donors influencing each other support the premise that the hematopoietic system takes the lead on educating engrafted NK cells. Thus, in contrast to KIR repertoire formation, which we found was independent of the HLA microenvironment, education of NK cells is markedly influenced by the presence of non-cognate HLA within the same recipient. Interestingly, a contamination as small as 5% with cells expressing the non-cognate ligand, as is the case in some mixed reconstituted huNSG mice, was enough to disrupt education. This phenomenon is also observed in the murine system, however only at higher frequencies, which suggests that the human system may be more sensitive (Johansson and Höglund, 2004; Johansson et al., 1997). The process that abrogates education and leads to reduced responsiveness toward targets is commonly referred to as disarming. Curiously, the disarming effect non-cognate HLA-ligands had on education was partially rescued in the case of KIR2DL2/3-positive NK cells in mixed reconstituted mice. It therefore appears that the presence of the low affinity binding ligand HLA-C2 in trans was sufficient to prevent disarming of HLA-C1 donor derived KIR2DL2/3^{+} NK cells (Pende et al., 2009). It currently remains to be uncovered which leukocyte populations are able to manipulate education and mediate disarming of NK cells. Furthermore, additional investigation is required to resolve the physiological role such disarmed NK cells play. As observed in our study, non-educated NK cells maintained self-inhibition and a dampened response towards targets expressing specific HLA-ligands, which might assist in mediating NK cell tolerance in mixed reconstituted huNSG mice.

Intriguingly, it has been shown that hyporesponsive NK cells are unable to counteract infiltration by HLA-negative tumors, yet have proven efficient at resolving murine CMV infection (Orr et al., 2010). Similarly, we found that in mixed reconstituted huNSG mice, where the majority of NK cells present are uneducated, viral loads upon EBV infection were reduced. This was seen both in blood and spleen at endpoint as well as indirectly as a measure of protection by a decreased accumulation of CD8^{+} T cells, i.e. decreased CD8^{+} T cell lymphocytosis. We were able to show that the improved control of EBV was mediated by NK cells as there were no discernable differences in the composition of the CD8^{+} T cell compartments (in terms of effector and memory differentiation) between single and mixed

reconstituted animals but the depletion of NK cells decisively led to a loss of protection as observed before in mixed reconstituted huNSG mice.

5.4 NK cells in hematopoietic cell transplant settings

Our results pose the question of how co-reconstitution of different donors in the setting of hematopoietic transplantation could influence disease outcome. Recent studies have underlined the value of NK cells as a means of immunotherapy in allogeneic hematopoietic cell transplantation. In haploidentical transplantation KIR-HLA mismatching in the graft-versus-host direction (i.e. presence of HLA-C1, -C2 or Bw4 in the donor but missing in the recipient) lead to NK cell-mediated donor-versus-recipient alloreactivity or so called graft-versus-leukemia (GvL) effects (Elliott and Yokoyama, 2011; Ruggeri et al., 2002). Mature NK cells that have developed from engrafted cells exhibit a NK cell repertoire, which mirrors that of the original donor. Moreover, these cells seem to be immediately educated, as they are able to mount an alloreactive response that kills recipient leukemic cells (Velardi et al., 2009). This is likely facilitated by their development in the bone marrow where they are primarily exposed to a microenvironment that is dominated by donor HLA from the transplanted hematopoietic cells. Thus, the repertoire of engrafting NK cells is educated to be both self-tolerant as well as recipient alloreactive, allowing them to react to missing-self on recipient targets. When looking at the functional capacity of reconstituting NK cells after unrelated donor or umbilical cord transplant, both cytotoxicity and cytokine production could be observed in NK cells educated by donor HLA (Foley et al., 2011). Interestingly, in HLA-matched transplant patients NK cells that did not express receptors for self-HLA remained hypofunctional after reconstitution. This emphasized the advantage of mismatched transplants, where the breaking of NK cell tolerance results in beneficial clinical outcome for patients. Furthermore, it was observed that NK cell differentiation occurred independently of education by self-HLA ligands, as is the case in healthy individuals (Björkström et al., 2010).

Acute myeloid leukemia (AML) is a condition, where in patients that have undergone haploidentical hematopoietic cell transplantation, the involvement of reconstituted NK cells is key in controlling the outcome of disease (Pietra et al., 2015). Alloreactive NK cells have been implicated in eradicating AML tumor cells, favoring engraftment, protecting from graft-versus-host disease (GvHD), lowering relapse rates

and greatly improving event-free survival, as demonstrated by data from multiple clinical and preclinical studies (Velardi et al., 2009). Also, in children with acute lymphoblastic leukemia (ALL), NK alloreactivity was shown to lower the risk of relapse (Moretta et al., 2011). Protection from ALL could additionally be improved when alloreactivity was exerted by NK cells of maternal donors primed by exposure to fetal antigens during pregnancy and thus able to cooperate with memory T-cell immunity against the paternal HLA haplotype (Stern et al., 2008).

Furthermore, transplantation of hematopoietic cells from unrelated donors was not improved when recipient and donors were KIR-ligand mismatched with only a few studies reporting improved GvL effects {Velardi:2008hv}. In contrast, KIR-ligand mismatch in the graft-versus-host direction during single unit cord blood transplants significantly reduced the incidence of relapse and improved leukemia-free survival in AML {Willemze:2009ib}. However, nowadays common practice is the administration of double unit transplants of which one donors tends to prevail during engraftment (Gutman et al., 2010; Hashem and Lazarus, 2015). When KIR-ligand mismatching was studied in the dominant engrafting unit, no beneficial effect could be documented on GvHD, transplantation-related mortality, relapse or survival (Brunstein et al., 2009).

Interestingly, in the transplantation setting the role of T cells is only poorly assessed, since depletion or naivety of transplanted T cells does not allow for a rapid contribution during engraftment. Therefore, transplantations combining adoptive transfer of T regulatory and effectors cells are of great relevance. Add-back of T cells was shown to enhance recovery of NK cells that were donor-versus-recipient alloreactive and single KIR positive (Di Ianni et al., 2011). The role of activating KIRs in transplantation has also been the focus of several studies. Consistent with previous evidence that NK cells may be instrumental in limiting GvHD, NK cells expressing KIR2DS1 efficiently kill allogeneic KIR-ligand-mismatched T cell blasts and DCs (Sivori et al., 2011), which suggests that they are important in patients receiving a KIR ligand mismatched haploidentical transplantation. In addition, data from large cohort studies indicated that the presence of activating KIRs and activating KIR genes in donors could support relapse protection and survival in AML (Cooley et al., 2010; Stringaris et al., 2010).

These interesting findings have encouraged trials that aim to eradicate AML by administering alloreactive NK cells. One such strategy is the infusion of haploidentical NK cells following lymphoablating immunotherapy. The adoptive transfer was well

tolerated and some patients showed long-term persistence of alloreactive NK cells in peripheral blood and bone marrow. This course of anti-leukemic treatment could be especially interesting for candidates who do not qualify for hematopoietic cell transplantation (Curti et al., 2011; Miller et al., 2005; Rubnitz et al., 2010). Another trial involves administration of anti-KIR antibodies, which block the recognition of self-HLA-C by inhibitory KIRs. In mice, this approach has produced encouraging results, leading to increased NK cell-mediated killing of targets, while maintaining self-tolerance and allowing for NK cell education (Romagné et al., 2009). These encouraging results may pave the way for *in vivo* therapies using antibodies to activate endogenous NK cells that can clear leukemia and other malignancies.

Lastly, the risk of CMV reactivation in hematopoietic cell transplantation patients induced by conditioning regimens is still a great threat and a major cause of treatment related mortality. To date, several studies have confirmed the existence of long-lasting memory-like NK cells in CMV-seropositive individuals (Béziat et al., 2011; Lopez-Vergès et al., 2011). Thus, transfer of memory NK cells from seropositive donors may help to keep reactivation at bay, as data from mice suggest (Sun and Lanier, 2011). In conclusion, NK cells are becoming attractive therapy tools, since unlike T cells, they do not induce GvHD and are able to target both leukemic malignancies and viral diseases (Elliott and Yokoyama, 2011; Ruggeri et al., 2002).

Taking into account all this knowledge, it is highly important to understand the interplay of KIR-ligands with their cognate receptors during engraftment to advance current therapeutic approaches. In addition, sustainable co-reconstitution could be assessed as a viable option to give patients an advantageous edge in controlling herpesvirus infections such as EBV and possibly HCMV. While the mechanism by which uneducated NK cells targets EBV is still elusive and warrants further investigation, a potential explanation might be found in the mismatching of the donors, such that mismatched EBV-transformed B cells might prove more susceptible to NK cell lysis, while the response to their matched counterpart would still be dampened by KIR inhibition (Yu et al., 2007).

PART II – INTERACTIONS OF NK CELLS WITH PDCS

5.1 NK cells enhance pDC survival despite cytotoxic IS phenotype

As interactions of NK cells with their surroundings are vital to their immune response, our second study involved the in-depth study of NK cell-pDC interactions. Curiously, the interactions we found showed hallmarks of a cytotoxic synapse, as accumulation of actin at the IS in NK cells was observed (Orange, 2008). However, pDCs were not found to be killed as seen by the lack of perforin polarization at the IS and the distance of the synaptic cleft that was comparable to that found in regulatory synapses (Barreira da Silva et al., 2011). In addition, pDCs even seemed to show enhanced survival in the presence of NK cells when lacking other persistence-boosting agonists. This correlates with data from a previous study, where NK cells were shown to functionally mature pDCs with no trace of cell lysis to be found (Gerosa et al., 2005). Moreover, NK cells also induce a positive feedback loop in pDCs towards oligodeoxynucleotides (ODNs), making them better responders and type I IFN producers (Gerosa et al., 2005).

5.2 Direct cell-to-cell contacts facilitate NK cell-pDC interactions

The accumulation of IFN signaling receptors and STAT signal transducers at the synapse suggest that the observed IS could aid in efficient signal transmission during infection. This would attribute similar functions to the IS of pDCs with NK cells as reported for the one with cDCs (Barreira da Silva et al., 2011). Indeed, results from Gerosa et al., showing that as little as 0.2% of pDCs were sufficient to transfer a type I IFN signal suggest that a specific means of interaction between NK cells and pDCs should exist to mediate such efficient cross-talk (Gerosa et al., 2005). Although no immediate role of the NK-pDC synapse could be reported, because transwell conditions performed comparable with respect to NK cell activation to conditions allowing direct cell-to-cell contact (Gerosa et al., 2005), the accumulation of receptors as mentioned above strongly hints toward a functional relevance of direct cell contact. In addition, the observation that low levels of type I IFN production upon poly(I:C) stimulation are able to mediate NK cell cytotoxicity too, might be explained by the well-organized capture of secreted type I IFNs via the IS (Gerosa et al., 2005). Furthermore, we could confirm the

involvement of STAT1 signaling during pDC-mediated NK cell induction as we were able to show polarized accumulation of phosphorylated STAT1 at the IS. As signaling via STAT1 has been implicated in cytotoxicity as well as accumulation and proliferation of NK cells (Nguyen et al., 2002), the enhanced cytolytic capacity of pDC-induced NK cells fits nicely with the phosphorylated STAT1 immunofluorescence images. Thus, we could demonstrated the preferential accumulation of STAT1 at the IS, which could facilitate the IFNα/β-STAT1-IL-15 signaling cascade, enhancing both NK cell cytotoxicity and survival at low levels of immune activation.

In keeping with data from Gerosa et al. that the HLA-DR-positive lymphocyte population induces CD69 upregulation on NK cells, we could also show that our purified pDCs displayed elevated expression levels of HLA-DR and were able to phenotypically and functionally mature NK cells as seen by upregulation of HLA-DR and CD69 (Gerosa et al., 2005). Curiously, while we did see expression of TNFα in stimulated pDCs the presence of NK cells markedly reduced the detected secretion levels possibly indicating an uptake by the present NK cells.

Thus, in microenvironments where IFNs could be scavenged by other cells, the formation of an IS between NK cells and pDCs might be important to ensure the efficient stimulation and response of NK cells. In fact, this might be particularly useful in non-productive viral infections where pDCs are only recruited in small numbers and therefore low amounts of type I IFNs are secreted and need to be directed efficiently toward NK cells (Gerosa et al., 2005). Moreover, as NK cell memory receives growing attention, the sufficient activation of NK cells towards vaccines could ensure a robust response toward a later challenge. In addition, targeting of NK cells through cytokines such as GM-CSF and IL-2 is being tested as a means to eradicate EBV-induced malignancies (Baiocchi et al., 2001). Thus, understanding activation of NK cells through IFNα/β could be important to design therapies taking advantage of host intrinsic pathways.

CHAPTER SIX – REFERENCES

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CHAPTER SEVEN – ACKNOWLEDGEMENTS

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CHAPTER EIGHT – CURRICULUM VITAE

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Date of Birth: 14 March 1988
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EDUCATION

2011 – 2016	**PhD at the Institute of Experimental Immunology, University of Zurich** Doctoral thesis: *Monitoring the effects of the HLA haplotype on NK cell KIR repertoire development*
2009 – 2011	**Master of Science ETH Zurich**, Major: Microbiology & Immunology Master thesis: *The immunological synapse between human plasmacytoid dendritic cells and NK cells* (overall GPA of 5.69 on a scale of 1 to 6, where 6 is the best grade possible)
2006 – 2009	**Bachelor of Science ETH Zurich**, in Biology (overall GPA of 5.14 on a scale of 1 to 6, where 6 is the best grade possible)
2002 – 2006	Grammer school in St.Gallen, Major: Natural Sciences (overall GPA of 5.17 on a scale of 1 to 6, where 6 is the best grade possible)

RESEARCH EXPERIENCE

2010 – 2011	**University of Zurich**, Institute of Experimental Immunology Project: *The immunological synapse between human dendritic cells and NK cells* Supervisor: Prof. Dr. Christian Münz
2010 – 2010	**University of Zurich**, Institute of Experimental Immunology Project: *The effect of NK cell depletion on the course of Epstein-Barr virus infection in humanized mice* Supervisor: Prof. Dr. Christian Münz
2009 – 2010	**ETH Zurich**, Institute of Microbiology Project: *Establishing a baculovirus expression system for RegIIIβ* Supervisor: Prof. Dr. Wolf-Dietrich Hardt

WORK EXPERIENCE

2010-2011	**Employee at the Chemistry Biology Pharmacy Information Center ETH Zurich** *- evening supervision of the library and all including operations*
2007- 2010	**Teaching assistant at the Institute of Integrative Biology ETH Zurich** *- leading a group of students on botanical excursions*
2009	**Teaching assistant at the Institute of BioEngineering ETH Zurich** *- part of the assistant team in charge of teaching basic biology to engineers*
2008 – 2009	**Translation of infomails for FMS AG** *- translation of company newsletter from German to English*
2005	**Summer job and translations at Bühler AG Uzwil** *- data processing and translation of correspondence from German to English*

LANGUAGE SKILLS

German / English	**Mother tongue**
French	Limited working proficiency
Chinese	Elementary proficiency
Portuguese	Elementary proficiency

COMPUTER SKILLS

MS Office	Experienced user of Word, Excel and Power Point
Adobe Suite	Proficient in image and text editing using Photoshop, lnDesign and Adobe Illustrator
imageJ	Skilled at image processing and analysis
FlowJo	In depth knowledge of flow cytometry data analysis
Prism	Expert understanding of graphic representation of data and statistical analysis

HOBBIES & INTERESTS

Sports	Jogging, Hiking, Fitness
SLRG	Swiss Life Guard Association
Travelling	South East Asia, Europe, South Africa, USA

CHAPTER NINE – DECLARATION

Herewith I declare that I have written this thesis myself and have only used the aforementioned references.

Zürich 14.04.2016

Vanessa Landtwing, Zürich, Switzerland